An investigation into selenium metabolism with reference to the interactions between vitamin E, selenium and other trace elements.

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AN INVESTIGATION INTO SELENIUM METABOLISM WITH REFERENCE TO THE INTERACTIONS BETWEEN VITAMIN E, SELENIUM AND OTHER TRACE ELEMENTS

by

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ABSTRACT

OLABISI ESTHER COKER

AN INVESTIGATION INTO SELENIUM METABOLISM WITH REFERENCE TO THE INTERACTIONS BETWEEN VITAMIN E, SELENIUM AND OTHER TRACE ELEMENTS

Selenium has been shown to be essential for several animal species and, although essentiality has not been proven for Man, there are several lines of reasoning to suggest it.

The recent discovery that selenium is an essential constituent of the enzyme glutathione peroxidase, which is found in the tissues of many animal species and has also been demonstrated in human erythrocytes, suggests that selenium may also be an essential element for Man. In addition, some of the urinary metabolites of selenium produced by man are the same as those produced by the rat, and the proven essentiality of selenium for the rat indicates that the same may be true for man.

In the work described in this thesis, the effects of alterations in the dietary levels of selenium and vitamin E on the metabolic route for the detoxification of acute levels of selenium have been examined in the rat. In addition, the activity of dietary conditions, and correlation between enzyme activity and the concentration of selenium in liver was studied. All experimental methods and enzymic assays have been described in detail.

The effect of silver, which causes selenium deficiency symptoms in vitamin E-deficient rats, on glutathione peroxidase activity and concentration of liver selenium was investigated in an attempt to explain gross, physical manifestations by changes in parameters of selenium metabolism. In the presence of toxic levels of dietary selenium, the effect of silver on selenium detoxification was investigated.

Finally, the short-term uptake of ^{75}Se in various tissues of rats given vitamin E, selenium and toxic metals such as mercury, cadmium and silver, was measured and compared with control rats in order to begin to explore the nature of these elements with selenium and vitamin E.

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PART I

HISTORICAL INTRODUCTION

CHAPTER 1

SELENIUM AS AN ELEMENT

Selenium was formally discovered by Berzelius (1818) when he noticed a red slime formed during the oxidation of sulphur dioxide from copper pyrites. At first thought to be a compound of sulphur and tellurium (already discovered by Klaproth in 1798), it was later shown by Berzelius to be a new element, closely resembling tellurium, and he gave it the analogous name "selenium" (selene (Greek), the moon), because of the appearance of one of the allotropic forms of the element, known as grey selenium. The actual discovery may, however, date to the fourteenth century work of Arnold of Villanova, who reported that a red sulphur deposit ("sulfur rubeum") was formed from oven chamber walls after vapour from crude sulphur had been condensed.

A. OCCURRENCE

Selenium ranks seventieth among the elements in order of abundance, and it comprises approximately $10^{-5}\%$ of the earth's crust (Rosenfeld and Beath, 1964). The element occurs widely distributed in igneous-type rocks and even in sea water. There are, however, very few ores which are primarily composed of selenium compounds, and most of the earth's selenium occurs in small amounts in pyrites and other sulphicle ores and in copper-bearing deposits. The production of selenium, therefore, is usually a by-product of the mining of other elements, primarily copper, zinc, nickel and silver. It is also obtained from sulphur ores and in uranium purification.

Among the naturally occurring selenium compounds are:

(a) clausthalite (PbSe) which is isomorphous with and closely resembles galena.

(b) naumannite - a similar ore in which silver is predominant.

(c) tiemannite (HgSe)

(d) croolcesite($(Cu,Tl,Ag)_2Se$)

(e) chalcomenite ($CuSeO_3 \cdot 2H_2O$)

Classification of Selenium (Table I-1)

The element is located between sulphur and tellurium in Group VIB of the Periodic Table and is in the fourth period between arsenic and bromine. Selenium has been classified both as a metal and as a non-metal since it lies between the non-metals oxygen and sulphur and the metals tellurium and polonium by group, and between arsenic, a metal and bromine, a non-metal, by period. Its atomic weight is 78.96, a value based on the chemical analysis of silver selenide Ag_2Se (Hönigschmid and Kapfenberger, 1933) and which has been confirmed subsequently.

Six stable isotopes have been isolated (Brasted, 1961) and are, in order of abundance, of mass 80, 78, 82, 76, 77 and 74. In addition, several unstable isotopes, including 70, 72, 73, 75, 79, 81, 83, 85, 86 and 87 have been identified.

The atomic number of selenium is 34, the electronic configuration being:

K	L	M	N
$1s^2$	$2s^2\ 2p^6$	$3s^2\ 3p^6\ 3d^{10}$	$4s^2\ 4p^4$

It is markedly similar to sulphur ($3s^2\ 3p^6$) in its chemistry and its primary oxidation states are -2,0, +2, +4, and +6. However, it may be that the clue to the biological role of the element may lie rather in its differences from sulphur than in its similarities to it (see Section C of this chapter).

B. PHYSICAL PROPERTIES

SOLID SELENIUM

Selenium exhibits several allotropic forms, all of which are stable at room temperature. The following are generally recognised as the

TABLE I - 1

PORTION OF THE PERIODIC TABLE SHOWING THE LOCATION OF SELENIUM

PERIOD	GROUPS			
	IVB	VB	VIB	VIIB
2	6 C 12.01	7 N 14.01	8 O 16.00	9 F 19.00
3	14 Si 28.09	15 P 30.97	16 S 32.06	17 Cl 35.45
4	32 Ge 72.59	33 As 74.92	34 Se 78.96	35 Br 79.90
5	50 Sn 118.7	51 Sb 121.8	52 Te 127.6	53 I 126.9
6	82 Pb 207.2	83 Bi 209.0	84 Po 210	85 At 210

separate allotropes of selenium, although each may occur in varying forms:

1. Amorrhous Selenium (α selenium)

Amorphous selenium may be obtained by the reduction of cold aqueous solutions of selenious acid with hydrazine, sulphur dioxide, hydrogen selenide, etc. The red particles thus obtained form a colloidal suspension. When heated above 60°, colloidal amorphous selenium crystallizes into colloidal hexagonal selenium.

2. Crystalline Selenium

Selenium is known to occur in three crystalline forms - α- monoclinic, β- monoclinic and hexagonal crystals. The monoclinic forms have a puckered ring conformation and are often referred to as red (α) or dark red (β) selenium. The hexagonal form, comprised of helical Se chains, is often called gray selenium.

Preparation: Evaporation of a saturated solution of selenium in carbon disulphide usually yields both α- and β- monoclinic forms. The α crystals are red in colour and have flat, hexagonal shapes whereas the β- form crystals are needle-like or prismatic and coloured deep red. Manual separation can, therefore, be achieved.

Hexagonal selenium is usually prepared by crystallization of amorphous selenium in the temperature range 70-210°, although larger crystals can be grown under high pressure crystallization.

The hexagonal form is the most stable; when heated above 110° both monoclinic forms transform to hexagonal selenium.

Electrical Properties

The amorphous forms of selenium are insulators, but the stable metallic modification is a relatively good conductor, the conductivity of which increases rapidly with temperature. It is now recognised that selenium falls within the class of semi-conductors and, as a general rule,

metallic impurities decrease, while non-metals increase, selenium conductivity.

The resistance of metallic selenium also decreases when it is illuminated by visible light or other radiations i.e. photoconductivity. Such properties have led to the use of selenium and its compounds in photocell devices as well as in xerography.

C. CHEMICAL PROPERTIES

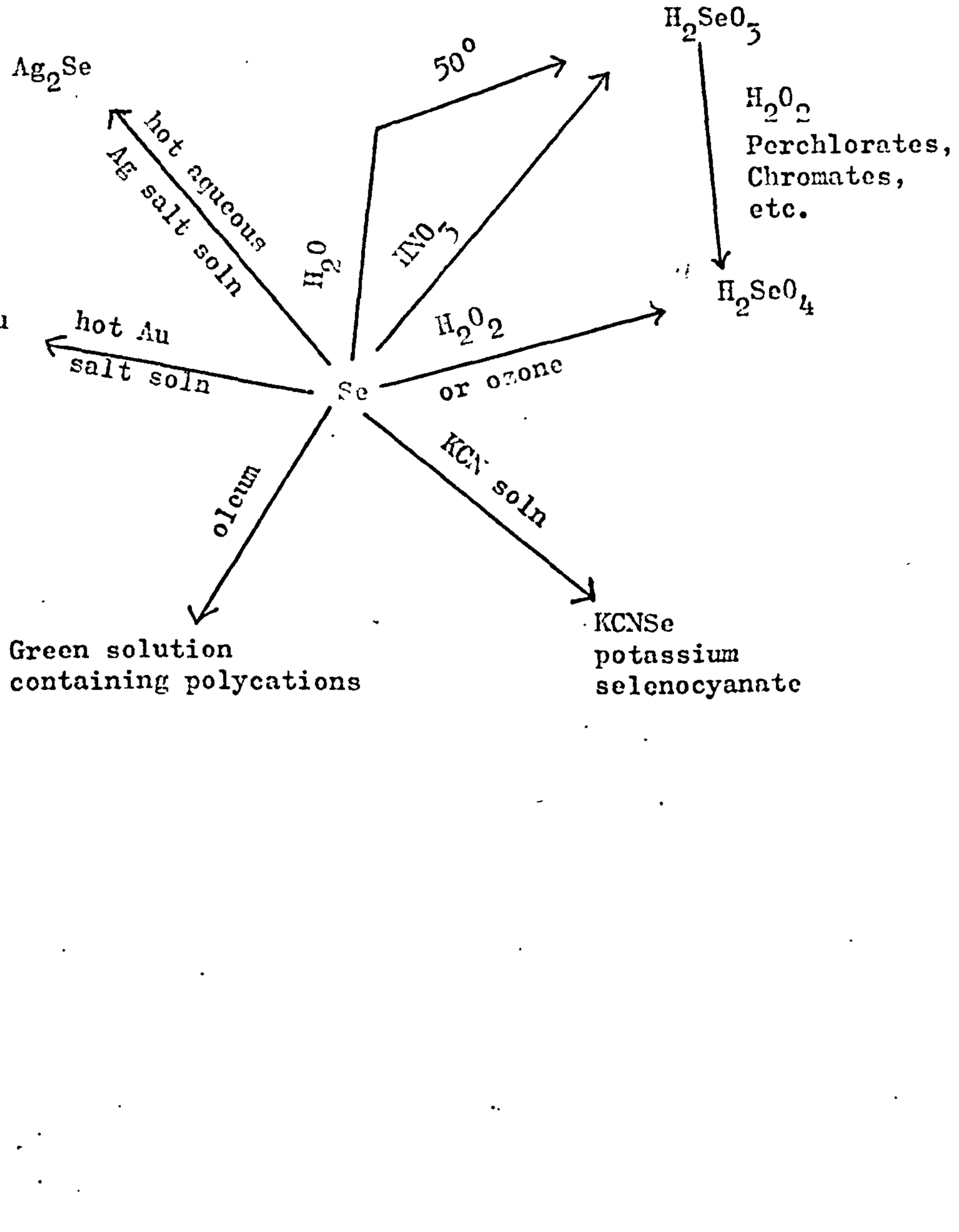
Selenium shows purely non-metallic chemical properties. Its compounds are usually covalent and there is a tendency in valency to achieve the inert gas configuration of krypton. It is not attacked by non-oxidising acids but, on heating, it will react with concentrated H_2SO_4 and HNO_3.

The stable gray form of selenium does not dissolve in water, but freshly precipitated amorphous selenium reacts with warm water (50°) to form selenious acid and hydrogen (Montignie, 1934). The element also dissolves in dilute alkali solutions giving red solutions which probably contain polyselenides.

The properties of selenium are, in general, intermediate between those of sulphur and tellurium. At high temperatures, selenium is able to displace sulphur from sulphides and, in a few biological systems, selenium may sometimes substitute for sulphur without loss of biological activity (Mukai et al, 1973; Mudd and Cantoni, 1957; Shift, 1961).

Other chemical properties of selenium are summarised in Fig I-1. When the chemical and physical characteristics of selenium and sulphur are considered, the similarities in the properties of the two elements are easily discernable; for example, the two elements have similar electronic configurations in their outermost valence shells although the 3d shell of selenium is completely filled. In addition, the sizes of the

CHEMICAL PROPERTIES OF SELENIUM



atoms of both elements are similar whether they are in the covalent or ionic state. (Covalent radius, Å :- S = 1.04; Se = 1.17; Ionic radius Å of M^{-2} :- S = 1.90; Se = 2.02; Levander, 1974). Further areas of similarity include the bond energies, ionization potentials and electron affinities of the two elements.

It is apparent, however, from a study of the systems <u>in vivo</u>, that although selenium and sulphur have many chemical and physical properties in common, they are seldom able to substitute for one another in bilogical systems. One reason for this might be that postulated by Rosenfeld and Beath (1964), who stated that Se in selenite tends to undergo reduction, whereas sulphur in sulphite is generally oxidised, a difference which can be represented by the equation: $H_2SeO_3 + 2H_2SO_3 \longrightarrow Se + 2H_2SO_4 + H_2O$. This chemical difference is also reflected in the metabolic fate of the two oxyanions (selenite and sulphite) since in mammals, selenium compounds generally tend to be reduced (Levander, 1974) while sulphur compounds tend to be oxidised.

Another chemical distinction between selenium and sulphur is in the relative strengths of the acids H_2Se and H_2S (Levander, 1974). Although the oxyacids of selenium and sulphur are of similar strength (pKa H_2SeO_3 = 2.6; pKa H_2SO_3 = 1.9), H_2Se (pKa 3.8) is a much stronger acid than H_2S (pKa 7.0). This difference in acidic strength is reflected in the dissociation pattern of the selenohydryl group of seleno cysteine compared to that of the -SH group of cysteine; the behaviour of the two groups was studied by Huber and Criddle (1967) and the pK of -SeH was found to be 5.24 while that for -SH was 8.25. Thus, at physiological pH, the sulphydryl group in cysteine or other thiols exists mainly in the protonated form while the selenohydryl group in seleno cysteine (or other selenols) is mainly in the dissociated form.

Therefore, although several similarities occur in the metabolism of selenium and sulphur, the physiological role of selenium may be better

understood when the chemical differences between the two elements are taken into account and the metabolic and chemical peculiarities of selenium are investigated.

CHAPTER 2

SELENIUM TOXICITY

A. Selenium toxicity in man

The increasing use of selenium and selenium compounds in industrial processes has drawn attention to the question of their toxicity. Selenium is used on a large scale in many manufacturing industries e.g. the production of coloured glass, pigments, photo-electric cells, rectifiers, transistors and to increase the machinability of steel. In addition, it is used as a catalyst or antioxidant in the production of rubber, plastics and xenographic plates. There are thus many industrial processes that can lead to exposure to toxic levels of selenium, in addition to excess selenium that may be supplied in foods from high Se areas, or inhaled from the atmosphere (Byers, 1937) in the vicinity of industrial plants that refine Se-containing sulphide ores.

Shapiro (1973) in a review, summarised the historical recognition of the symptoms of selenosis in man. Congenital defects and the loss of hair and nails in Columbia, South America, during the sixteenth century, is said to have been due to excess dietary selenium (Benavides and Mojica, 1959). Two centuries ago, Jacquim (1763) declared the fruit of the monkey pod tree (which is now recognised as seleniferous) to be toxic.

Although water supplies have not generally been considered to be a potential source of selenium toxicity, even in seleniferous areas, a report by the U.S. Bureau of Water Hygiene (1972) suggests that excessive amounts of selenium in soil may contaminate local water supplies. Smith *et al* (1936) were the first workers to carry out a systematic, epidemiological study of the chronic toxicity of selenium in man. Analysis of dietary selenium content and urinary selenium levels were carried out on one hundred and eleven families living in South Dakota, Wyoming and Nebraska, where farm animals were known to be suffering from selenium toxicity

("alkali disease"). Several signs and symptoms were attributed to excessive selenium intake; bad teeth, jaundice, chloasma, vertigo, chronic gastrointestinal disease, dermatitis, nail changes, arthritis, oedema and fatigue. Analysis of the selenium content of the food of these people revealed a daily selenium intake of 0.1 to 0.2 mgSe / kg body weight. Selenium was detected in the urine of 92% of the people tested and 45% of these contained 0.2 to 1.33 ppmSe.

Dental caries has also been suggested (Hadjimarkos 1961, 1969) as a symptom of selenium toxicosis; when the incidence of dental caries in children and the levels of urinary and dietary selenium were correlated, urinary selenium was found to be twice as high in those children with a high prevalence of caries. These results agree with the findings of Buttner (1963) who demonstrated an increased incidence of caries in rats fed high levels of dietary selenium. Other attempts to correlate a high incidence of caries with dietary selenium levels have, however, led to inconsistent (Muhleman and Konig (1964)) or marginally significant (Ludwig and Bibby, 1969) results.

In contrast to this uncertainty, acute and subacute selenosis caused by industrial exposure to selenium and its compounds is reproducible and generally severe in nature (Cerwenka and Cooper, 1961). Selenium may be inhaled as fumes or dust, or absorbed through the skin or gastrointestinal tract; marked irritation of the nasal, conjunctival and tracheobronchial mucosa occurs rapidly, which gives rise to coughing, wheezing, dyspnoea, chemical pneumonitis and pulmonary oedema. A low-grade fever with abdominal pain, nausea, vomiting and diarrhoea usually follows, with chronic dermatitis in exposed and unexposed areas of skin. The affected persons complain of nervousness, fatique, depression and pallor, but the most recognizable characteristic is the garlic-like odour of the breath and sweat caused by the excretory product dimethylselenide (see Chaper 8

of this thesis). A strong metallic taste in the mouth is commonly reported after selenium ingestion.

Toxicity of commonly used compounds

Sax (1968) listed over 20 industrial materials containing selenium as potentially hazardous. Among the substances most commonly encountered are:

Elemental selenium	-	only harmful when inhaled as dust or vapour. Irritates pulmonary mucosa and causes rhinitis and conjunctivitis (Dudley 1936; 1938; Clinton, 1947).
Hydrogen selenide (H_2Se)	-	highly toxic compound; odour is offensive but it induces olfactory fatigue causing insensitivity to its smell. Limit of exposure = 0.05 ppmSe or 0.2 mgSe/m^3air (Cerwenka and Cooper, 1961).
Dimethyl selenide $(CH_3)_2Se$	-	produces severe pharyngitis and bronchitis. Recurrence of symptoms on minimal re-exposure indicated an allergic state (Motley et al 1937)
Selenium oxychloride $SeOCl_2$	-	causes blistering on contact with skin followed by absorption of the selenium. 0.01 ml caused death when tested on rabbits, and severe burns on human skin (Dudley, 1938 a).
Inorganic selenites and selenates	-	cause skin irritation on contact. Reported to be associated with porphyrinuria (Halter, 1939).
Selenium dioxide (SeO_2)	-	forms selenious acid on contact with moisture which causes a haemorrhagic rash (Pringle, 1942). SeO_2 also causes conjunctivitis and painful irritation of skin.

Teratogenicity of selenium

Recently, Hadjimarkos (1970) has drawn attention to the possible teratogenicity of selenium and has urged that, in view of the many female workers employed in selenium-utilising industries, further investigation is needed. In a separate study, Robertson (1970) found that, of ten women using selenium in an industrial situation, five became pregnant during the period of study and four aborted; a full-term infant was born to another with a club-foot. These observations confirm earlier reports of the teratogenicity of selenium in other species: Franke et al (1936) observed occurrence of embryonic chick monsters on farms where seleniferous wheat was used as feed. Gruenwald (1958) studied the embryos of eggs produced by selenium-fed hens and found foetal tissue necrosis with subsequent retardation of development as an early manifestation of selenium toxicity. Similarly, Wright and Mraz (1965) found that 20µg Se injected into chick embryos prevented hatching, while 30µg caused death. Lesser doses resulted in dwarfness, shortened cranial viscera, shortened extremities and digital fusion.

Selenium as a carcinogen

Considerable dispute exists as to whether or not selenium is carcinogenic. This matter is of more than theoretical interest because of the widespread use of selenium in industry, the fact that selenium may be administered to animals destined for human consumption and because of the possible danger to millworkers and others employed in the feed-stuffs industry where selenium is used as an additive to prevent selenium deficiency in animals. Miller (1966) and Kraybill (1969) both list selenium as tumorigenic and carcinogenic; however, Frost (1967; 1972) suggests that there may be a confusion between toxicity and carcinogenicity.

In contrast to the reports of Miller and Kraybill, Mautner (1956) and Mautner and Jaffe (1958) have prepared the selenium analogue of 6-

mercaptopurine and have found it to be as effective as the sulphur parent compound in the inhibition of mouse leukemia L1210.

Similarly, Clayton and Baumann (1949) have observed a reduction in the incidence of induced hepatic tumours in mice by the addition of 5 ppm sodium selenite to the animals' diet. Shamberger (1969; 1970) and Riley (1968) using sodium selenide have also demonstrated a decrease in the number of chemically induced skin tumours when Na_2Se was fed to mice.

In a detailed study of parts of the United States and Canada, Shamberger and Frost (1969) reported that cancer death rates in 1965 were lower in areas where the average forage crop selenium was higher than 0.06 ppm than in localities where the level was lower than 0.05 ppm. The observed inverse relationship between selenium blood levels and cancer death rates suggested that an adequate selenium intake resulted in a diminished cancer death rate. Frost (1970) proposed that selenium may function in an anticarcinogenic manner, through either its association with -SH groups or possibly in protein synthesis.

B. <u>Selenium toxicity in animals</u>

The ingestion of seleniferous plants by livestock, with the consequent development of well-defined disorders, has been a problem to those concerned with livestock husbandry for many centuries. Probably the first report describing a disease syndrome resulting from the ingestion of seleniferous plants was that of Marco Polo during his travels to the Orient. He recorded that his horses were afflicted by a strange disorder largely characterised by the fact that they shed their hooves. Only recently has it become evident that this disorder is characteristic of a type of chronic selenosis. The existence of seleniferous areas in the United States was first reported in 1934 by Franke who carried out experiments in South Dakota on the toxicity to cattle and horses of grains and grasses grown in the area; his findings established that the

toxicity was due to the presence of selenium in these forages. In addition, an interbureau co-operative study by the United States Government found (Robinson, 1933) that the wheat which produced symptoms of the toxicity later shown to be selenosis, contained between 5 and 12 ppm of selenium.

The clinical signs and pathological changes produced by toxic quantities of selenium vary with the animal species, the age of the animal, the chemical form and quantity of selenium consumed or administered and the nature of the diet exclusive of selenium. Addition to the diet of other elements, such as arsenic (Levander,1972), silver (Diplock et al 1967); Part III, Chapters 13-16 of this thesis), mercury and cadmium (Parizek et al 1974) or of other dietary constituents such as linseed oil meal or sulphur amino acids (Levander,1972) also modifies the selenosis.

The toxicity is divisible into acute toxicity, which is rapidly fatal, and chronic toxicity. Acute poisoning is caused by the consumption of an excessive quantity of selenium in a short period of time and the liver is the organ that is most affected. It undergoes fatty degeneration (Smith, 1939) which is reversible if the exposure is short. When exposure is prolonged, cirrhosis of the liver develops, quickly followed by death. Acute poisoning is often recognised by the abnormal posture of the affected animal; it will walk unsteadily for a short distance and then stop and assume a curious stance with lowered head and drooping ears. Cyanosis of the mucous membranes, laboured breathing, diuresis and bloating are also usually seen. Death occurs in coma shortly afterwards, although the duration of the illness varies from a few hours to several days.

The chronic toxicity of selenium was classified into three categories by Rosenfeld and Beath (1964):

(1) "Blind Staggers" - caused by organic selenium compounds with or without small amounts of selenate, which are readily extracted by water from native seleniferous plants.

(2) "Alkali Disease" - produced in farm animals that have consumed plants or grain containing protein-bound selenium, presumably as seleno amino-acids, and thus relatively insoluble in water.

(3) Chronic selenosis - produced in experimental animals by the administration of selenite or selenate.

The "blind staggers" type of chronic selenium poisoning occurs in cattle and sheep feeding on moderate amounts of seleniferous plants of the genera Astrogalus, Xylorhiza, Stanleya and Oonopsis. Affected cattle show signs of central nervous system disorder, such as aimless wandering, circling, bumping into objects in their path and stumbling over obstacles that do not exist. There is a marked loss of appetite, paralysis and general loss of muscular tone. The symptoms in sheep are less characteristic.

"Alkali disease" occurs when livestock feed on seleniferous grains and grasses. The clinical signs in horses, cattle and swine (Franke et al, 1934) include retarded growth, emaciation, deformed hooves, loss of hair and arthritic disorders of the joints.

C. UPTAKE AND EXCRETION OF SELENIUM

1. Chronic Toxicity

When selenium is given orally as a water-soluble compound or in seleniferous grains and grasses containing the element in water-soluble form, the element is readily absorbed from the gastrointestinal tract. Rapid distribution throughout the body occurs, with greater concentration in the liver and kidneys. Moxon (1937) studied selenium uptake by dogs

and showed that a concentration of about 30 ppm in the dry matter from liver and kidneys was achieved, whereas the concentration in other organs did not exceed 5 ppm.

The major route of selenium excretion in most animals is via the urine. In ruminant animals, however, particularly sheep, a considerable portion is also eliminated in the faeces.

In a careful study in the mouse, Heinrich and Kelsey (1955) measured the uptake of ^{75}Se from a single dose of ^{75}Se-sodium selenite. Female white mice (15-24 g weight) were injected subcutaneously with 0.1 - 0.7 mg ^{75}Se per kg body weight. The animals were placed in glass metabolism cases; urine and faeces were separated and expired air was bubbled through a saturated solution of mercuric chloride (McConnell and Portman, 1952). At varying time intervals, the animals were killed by decapitation and exsanguinated. Heart, liver, lung, kidney, aliquots of the urine and of the mercuric chloride solution were placed in glass vials and ^{75}Se determined using a scintillation counter. The graphs in Fig. 2-1 were compiled from these readings and show the distribution of ^{75}Se in those tissues after injection of $Na_2{}^{75}SeO_3$.

From their results, Heinrich and Kelsey concluded that, when given a single sublethal dose of sodium ^{75}Se-Selenite, mice excrete about 83% of the dose within 48 hours. After only four hours, 36% is excreted via the urine, less than 1% in the faeces and about 3.5% in the expired air. After 48 hours, the urine is found to have removed 65.5% of the dose, the faeces contains 10.5% and 7.5% is lost through the expired air. Thus, between four and 48 hours after ^{75}Se administration, there is a rapid increase in the rate of selenium excretion and a simultaneous decrease in the levels of ^{75}Se retained by the tissues.

This study established, therefore, that when small amounts of selenium are administered, the main route of excretion is via the kidneys

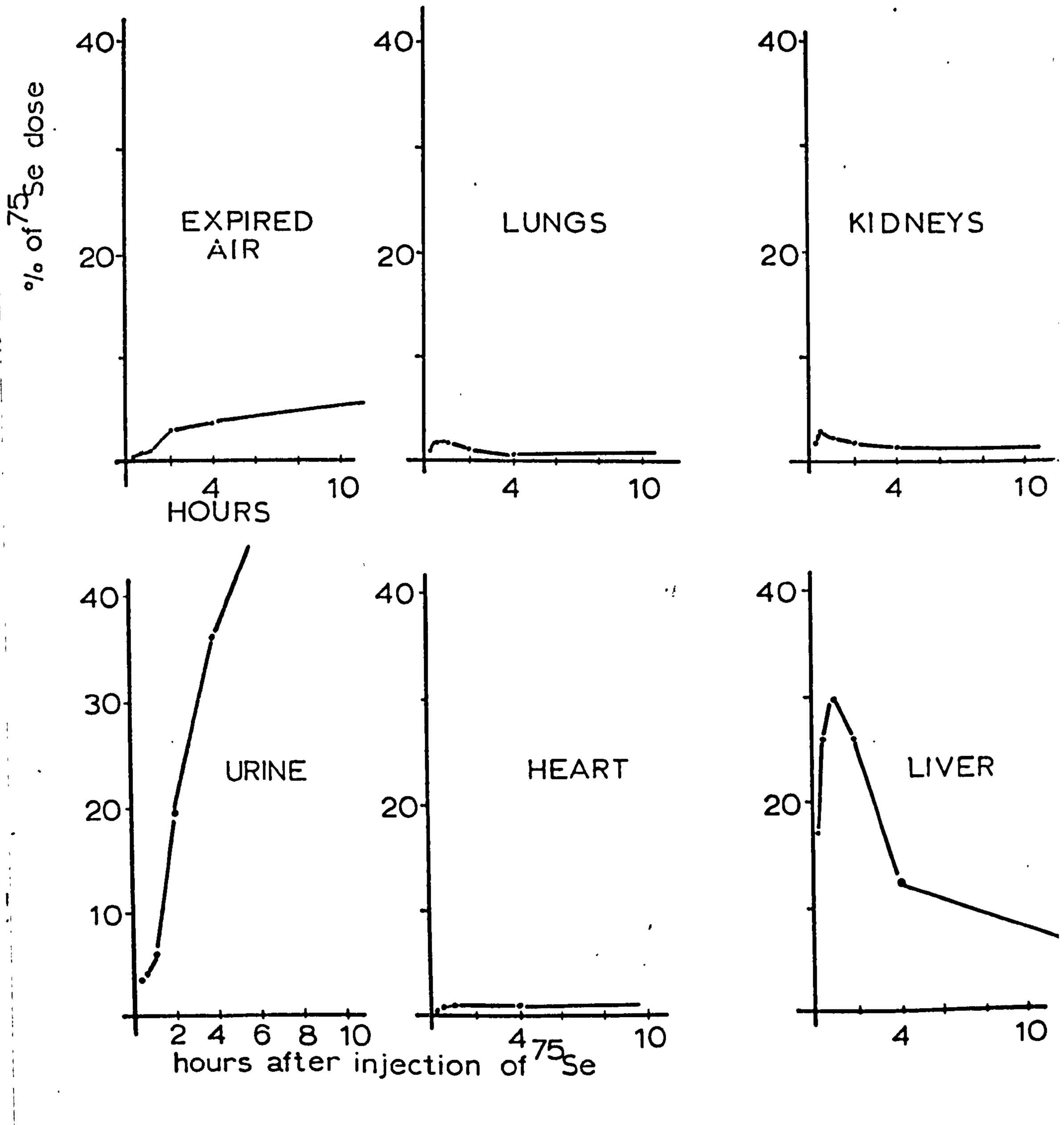


Fig. 2-1. (From Heinrich and Kelsey, 1955)

Graphs show the distribution of ^{75}Se in female white mice after subcutaneous administration of a dose (0.1-0.7 mg ^{75}Se/kg body weight) of ^{75}Se- sodium selenite. Urine was collected and ^{75}Se in expired air was trapped in $HgCl_2$ solution. The animals were killed at intervals of up to 48 hrs. after the injection. Each point represents the average of at least six analyses of the wet tissues.

in the urine. This finding has been confirmed by other workers since: Ganther (1965) in a study using much small doses (25 µg per animal) also found that the major route of elimination was by way of the kidney; but after the first 24 hours, only 29% of the administered selenium was excreted in the urine and 8% in the faeces. Similarly, Halverson et al (1962) fed rats a diet containing 5 ppm Se as selenite for a two week period. During this time, 51% of the ingested selenium was excreted in the urine and 12% in the faeces. The relatively large proportion of the administered ^{75}Se dose found in the faeces suggests that this route of excretion should not be overlooked. However, in an earlier study by Smith and co-workers (1936), similar amounts of selenium were found to be quantitatively eliminated in the urine of cats chronically poisoned with selenite, and the faecal route was not apparently used to any great extent. Whilst it is possible that changes in the nature of the intestinal microflora may cause changes in the proportion of a dose of selenium that is eliminated in the faeces, it should be noted that faecal excretion is not necessarily due to unabsorbed selenium or selenium that is taken up by the gut microflora before excretion. Ganther and Baumann (1962) showed that 20-30% of a dose of selenium was recovered in the faeces following the subcutaneous injection of selenite or selenate. Although significant amounts of selenium are exhaled through the lungs of animals given small chronic doses of selenium, this route of excretion is relatively uninportant; however, when larger doses of selenium are given, this route becomes of great importance (see Chapter 8).

When small amounts of selenium are administered daily over a long period of time, the tissues do not appear to accumulate the element to any great extent. Thus, the study of Munsell and co-workers in 1936 and that of Anderson and Moxon (1941) on the rat, showed that in rats given seleniferous wheat, storage of selenium reached a maximum after a few weeks and then plateaud off. In addition, Anderson and Moxon found

that the amount of selenium retained from a dose of constant size falls sharply after an initial period during which a large percentage of the dose is taken up by the tissues. Ganther, in work for his master's degree (1961), found that when a dose of 25 mg ^{75}Se is administered on four consecutive days, 63% of the initial dose was retained on the first day; on the second day, only 46% of the administered dose was retained and this figure had fallen to 40% and 33% by the third and fourth days respectively. While the remainder was predominantly excreted through the urine, some was also lost in the faeces.

This observation tends to suggest that the tissues can reach saturation with selenium; when this happens, the excretory mechanism becomes stimulated so that proportionately a larger amount of selenium is metabolised as time passes.

2. Acute Toxicity

The administration of small doses of selenium to laboratory animals produces similar uptake and distribution patterns as in sub-acute or acute dosage (McConnell, 1941). However, as the dosage of selenium is increased, the formation of volatile selenium and its elimination via the lungs becomes of increasingly greater significance (McConnell and Roth, 1966).

Long before selenium was recognised as the causative agent in blind staggers and alkali disease, Hofmeister (1894) reported that the breath of dogs given sodium selenite, had a garlic-like odour. He postulated that this volatile substance was dimethyl selenide. This phenomenon was first studied in detail by Schultz and Lewis (1940) who gave rats subcutaneous injections of 2.5 - 3.5 mg Se / kg body weight as Na_2SeO_3 and collected the respired gases for eight hours after the injection. The gases were passed through concentrated sulphuric acid and analysed using the selenium-codeine colorimetric assay (Horn, 1934;

Gortner and Lewis, 1939). Schultz and Lewis found that from 17% to 52% of the injected selenium was excreted within eight hours as a volatile compound which was absorbed by conc. H_2SO_4. Further, addition to the diet of methionine or choline chloride as potential methylating agents, did not increase the excretion of volatile selenium compounds. Two years later, McConnell (1942) published his findings on time-excretion studies of exhaled selenium in rats, using radioactive ^{75}Se as a tracer. Sodium selenate (Na_2SeO_4) was the compound used and it was given by subcutaneous injection. It was found that 3 to 10% of the dose was exhaled within 24 hours and about 75% of this amount was excreted during the first six hours. Thus, it appears that substantially less volatilization of a subacute dose of selenate occurs than of a similar dose of selenite. Other workers studying the effects of selenomethionine (McConnelland Roth, 1966) and seleniferous wheat (Olson et al, 1963) found that lower levels of pulmonary excretion followed the administration of these substances as compared to selenite.

As the quantity of administered selenium increases, the proportion excreted via the lungs also increases, and McConnell and Roth (1966) found this to be true in rats given either selenite or selenomethionine. On the other hand, Steinberg and Imbach (1967) confirmed this only with selenite, as they were unable to detect any pulmonary excretion of selenium in rats given selenomethionine. The difference in the observations of the two groups may be due to the sex of the animals and the mode of presentation of the selenium: McConnell and Roth used male rats and administered the selenomethionine subcutaneously while Steinberg and Imbach used female rats and administered the selenomethionine intravenously.

3. Physiological Levels

Early studies on the prevention of dietary liver necrosis in rats showed that the quantitative requirement for selenium for this purpose

was approximately 0.04 ppm when added to a Torula-yeast diet deficient in vitamin E. The nutritional requirements of other species for selenium have been determined and are found to be related to the vitamin E content of the diet. Scott (1973) discussed the nutritional experiments carried out using various animals and concluded that for chicks receiving a vitamin E-deficient diet, the selenium requirement is 0.08 ppm for prevention of exudative diathesis and 0.02 ppm for the prevention of pancreatic fibrosis. However, in diets containing the usual amounts of vitamin E present in normal practical feedstuffs (10-15 i.u/kg diet), the selenium requirement for chicks appears to be approximately 0.04 ppm.

Turkeys require more selenium than chicks; in turkey poults receiving a vitamin E-deficient diet, approximately 0.28 ppm Se is required to prevent gizzard and heart myopathy. A level of 0.18 ppm Se has been shown to be required when the diet contains normal practical levels of vitamin E. The requirement for the pregnant ewe appears to be 0.1 ppm to prevent white muscle disease in the progeny which is independent of dietary vitamin E; a similar amount is required by the pregnant cow to prevent muscular dystrophy in calves.

Scott (1973) considered that selenium levels in blood and tissues reflect the dietary level up to levels of dietary selenium that are slightly above the nutritional requirement. He fed graded levels of selenium (as Na_2SeO_3) to chicks and turkey poults and found that the amounts of selenium found in the blood, muscle, skin, liver and kidney increased until the diet contained about 0.4 ppm Se. Above this level the amount of selenium in the tissues tended to plateau. Parallel experiments with dietary selenium supplied in seleniferous wheat (0.67 ppm) showed that tissue levels increased above the plateau. Scott postulated that ingestion of inorganic selenium leads to the retention of only that amount which is capable of reacting with selenium binding sites (possibly cystine and other sulphur compounds). Any excess inorganic selenium over

that which can be found appears to be quickly excreted. When the dietary selenium is present as selenomethionine or selenocystine, direct deposition into the tissue proteins seems possible. Thus, considerably higher levels of selenium may be accumulated than when inorganic selenium is given.

Similarly, Ehlig et al (1967) found that lambs given a daily dose of 0.4 mg Se/400 g feed (1 ppm) as sodium selenite excreted 29 to 34% of the dose in the urine, whereas those given the same amount of selenium as selenomethionine excreted only about 23% of their daily doses in the urine. Thirteen days after initiation of the treatments, tissues from lambs given Se-methionine contained significantly more selenium than tissues from lambs given sodium selenite. These workers concluded that the greater retention of selenium by lambs given Se-methionine was due to a lower rate of urinary excretion by lambs given that treatment, than those given the sodium selenite treatment.

From his experiments on rats, Hopkins (1962) found that in animals given a diet low in both vitamin E and selenium, the retention of microgram doses of selenium was almost complete. Only 5% of a dose of 0.025 mg of Se was excreted within 24 hours in the urine and a similar amount in the faeces. Experiments in this laboratory using Se (Chapter 16) also confirm that selenium deficient animals retain more selenium - 75 than do those which have been receiving a diet adequate in the element.

Thus the uptake, retention and excretion of selenium all depend on the dietary or background level of selenium and on the type of selenium compound which is given to the animal.

CHAPTER 3

SELENIUM AS AN ESSENTIAL MICRO-NUTRIENT

The earliest recorded observation of necrotic liver degeneration in rats was by Weichselbaum (1935) who attempted to maintain animals on a cystine-free diet; he stated that this disease bore no relationship to fatty liver or cirrhosis. In confirmation of this, Daft et al (1942) showed that whereas choline completely protected against cirrhosis of the liver, it had no effect upon necrotic liver degeneration; cystine, on the other hand, completely prevented liver necrosis but did not prevent cirrhosis. Schwarz (1944) reported that rats given a diet low in both vitamin E and sulphur amino acids died of necrosis of the liver, and these findings have subsequently been confirmed by other workers (Himsworth and Lindan, 1949; Goettsch, 1950). Seven years after his first communication on this subject, Schwarz (1951) showed that a factor, present in baker's yeast, brewer's yeast and certain other natural products, was also capable of preventing dietary necrotic liver degeneration. He termed this unidentified nutrient "factor 3". Subsequent studies by Schwarz and co-workers (1954) showed that although baker's yeast was an excellent source of factor 3, Torula yeast was totally devoid of the antinecrogenic activity. Torula yeast (Torulopsis utilis) which is cultured on the sulphite waste liquors of the paper pulp industry, was, therefore, used as the source of protein in the diets used by Schwarz to study factor 3 and to assay concentrates of it.

During the fractionation of factor 3 from acid hydrolysales of natural materials, Schwarz and Foltz (1957) obtained two chemically similar substances with factor 3 activity. These were designated as α- and β - factor 3. α-factor 3 is water soluble, strongly anionic and stable to oxidation, but sensitive to reducing agents. Further fractionation of it led to semicrystalline products which, on alkaline treatment, produced a characteristic garlic-like odour. Since it was

known (Munsell et al, 1936) that the breath of cattle consuming plants containing high selenium levels derived from the seleniferous soils on which they grew had a garlic-like odour, Schwarz and Foltz investigated the possibility that the factor 3 preparations might contain selenium. Analyses showed that substantial amounts of the element were indeed present and that the biological activity, in the antinecrogenic assay of the preparations, was highly correlated with the selenium content. Shortly after this, sodium selenite was shown to be completely effective in preventing dietary liver necrosis in rats given a diet based on Torula yeast as the protein source. Schwarz and Foltz (1957) showed that the dose of selenium needed to afford complete protection was less than 1% of the dose of 300-400 µg/100 g of ration which had previously (Munsell et al, 1936) been shown to produce chronic toxicity. They went on to say:

"In comparison with previously reported values for vitamin E and cystine (Schwarz, 1954), selenium is exceedingly effective in the prevention of necrotic liver degeneration. When applied in the form of sodium selenite, it is approximately 500 times as active as vitamin E and 250,000 times as active as L-cystine. The element has been shown to be constantly present in tissues of higher animals (Underwood, 1956). It can be inferred from our results that selenium is an essential trace element".

During the early 1950's, concurrently with the studies by Schwarz and his associates on dietary liver necrosis in rats, studies were being conducted by Scott and co-workers at Cornell University on an unidentified factor required for the prevention of "exudative diathesis" in chicks. This name had been given by Dam and Glavind (1938) to a nutritional condition in chicks which is characterised by accumulation of subcutaneous fluid throughout the bodies of chicks and turkey poults affected by the disease. The largest amounts of exudate are found beneath the skin covering the breast and abdomen, and small haemorrhages are usually seen in the musculature under-lying the largest accumulations of the fluid.

The plasma of affected chicks is seen to be depleted of protein, particularly of albumin (Goldstein and Scott 1956) and the exuded fluid has a composition similar to the blood plasma. Measurement of the electrophoretic mobility of the exudate and of plasma proteins gives similar results and it seems that the oedema is caused by an increase in the permeability of the walls of the blood capillaries; thus, Dam and Glavind (1940) showed that capillaries of chicks with exudative diathesis were more permeable to tryptan blue than those of normal chicks.

During the work at Cornell University, it was discovered that an unknown factor, present in baker's yeast was capable of sparing the vitamin E requirement for the prevention of exudative diathesis in chicks given a Torula-yeast based diet without added vitamin E (Scott et al, 1955). The similarity of this to the circumstances surrounding the investigation by Schwarz of dietary liver necrosis in the rat, led to a collaborative study which showed (Scott et al, 1957) that the potent Factor 3-active selenium, containing compounds of Schwarz were also capable of preventing exudative diathesis in the chick, while all preparations found by Schwarz to be devoid of Factor 3 activity also did not prevent exudative diathesis in vitamin E deficient chicks.

A study of the effect of selenium compounds upon exudative diathesis in chicks showed that 0.1 ppm of selenium as sodium selenite was effective in preventing the disease; also, selenocystathionine was shown to serve the same purpose. In addition, selenomethionine, seleno-cystine and selenocystathionine have all been shown to be approximately as effective as sodium selenite in preventing both dietary liver necrosis and exudative diathesis. (Scott, 1973).

In completely independent studies, Patterson et al (1957), using the Torula yeast diet of Scott and co-workers (1955), discovered that the unidentified factor which protected chicks against exudative diathesis

was present in the acid hydro-lysate of hog kidney but that the ash of acid-hydrolysed kidney was inactive. However, pre-treatment with calcium oxide, before burning led to retention of the activity. The activity of an alkaline ash and inactivity of an acid ash suggested that the activity of the factor in kidney was due to an element which forms a volatile inorganic acid. Among the elements forming such acids are arsenic, selenium and tellurium. Subsequent studies (Stokstad et al 1957) showed that both arsenic and tellurium were inactive, but that selenium given as sodium selenite was completely effective in preventing exudative diathesis in chicks.

CHAPTER 4

NUTRITIONAL DEFICIENCY OF SELENIUM IN ANIMALS

Selenium- responsive disorders occur in many species and they clearly illustrate that an inadequate supply of the trace element can cause vastly different deficiency symptons in various animals. Although liver necrosis is the most important lesion seen in the rat, it is but one pathological manifestation of a disorder which involves other organs as well. Almost all selenium-responsive diseases are associated with massive changes in parenchymal organs, and almost all of them are fatal. Schwarz (1976) compiled a list of deficiency symptoms in different species (Table 4-1), which will respond to selenium treatment. In chicks and other fowl, exudative diathesis is the predominant syndrome, (Patterson et al, 1957; Schwarz et al, 1957), but the most outstanding change seen in many species is muscular dystrophy.

1. Multiple Necrotic Degeneration in the mouse and dietary liver necrosis in the rat.

Dietary liver necrosis in the rat was described by Schwarz (1958) as "a case of widespread systemic injury which involves other tissues as well". If the diet which produces liver necrosis in the rat is fed to mice, multiple necrotic degeneration develops. Aside from liver necrosis, the heart muscle degenerates, muscular dystrophy develops, the kidneys are severely damaged and the pancreas undergoes severe atrophy. In multiple necrotic degeneration in the mouse, the heart lesion predominates and develops several weeks before other gross changes can be observed, and it accounts for 91% of the symptoms of multiple necrotic degeneration (Schwarz, 1958) as compared with 54% for the liver lesion.

In the rat, the liver is apparently the preferred site for massive necrosis. The liver lesion develops very quickly and kills the animal before other changes appear. In the rat therefore, selenium deficiency

TABLE 4 - 1 (Schwarz, 1976)

Selenium-Responsive Deficiency Symptoms in Different Species

(+) symptoms present (or absent (-)) only in severe cases of deficiency

	Growth Deficit	Liver Necrosis	Kidney Damage	Heart Muscle Degeneration	Muscular Dystrophy	Calcifications	Lung Hemorrhage	Pancreas Atrophy	Serum Protein	Exudative Diathesis
RAT	(+)	+	+	(-)	(+)	+	(+)	(+)	+	-
MOUSE	(+)	+	+	+	+		(-)	+	+	-
RABBIT		+					+			
MINK				+	+					
HOG		+			+					
SHEEP	(+)			+	+	+				
CATTLE				+	+	+				
CHICKEN	+				(+)			+	+	+
TURKEY	+				(+)				+	+
HORSE					+					

is usually assessed by the condition of the liver and the disease is described as dietary necrotic degeneration of this organ. It should be noted however, that degeneration of the intercorticomedullary zone of the kidney has been detected (Schwarz, 1958) in about 87% of all animals succumbing to liver necrosis; muscular dystrophy has also been observed on occasions.

Schwarz described dietary liver necrosis as being divisible into two phases:

1) a latent phase
and 2) a terminal phase

During the latent, prenecrotic phase, the liver appeared essentially normal, but metabolic disturbances were detectable and degeneration of the endoplasmic reticulum and mitochondrial swelling were seen in electron micrographs (Fite, 1954). These disturbances were characterised by a biochemical lesion which was manifested as an inability of liver slices and homogenates to maintain normal respiration <u>in vitro</u> for extended periods of time (see Chapter 5). Schwarz attributed this to the effects of a deficiency of Factor 3 or selenium on the enzyme lipoyldehydrogenase. Addition of vitamin E to homogenates <u>in vitro</u> protected the enzyme while selenium added similarly was inactive. Supplementation of the diet with selenium was not as effective as when vitamin E was used.

The <u>terminal phase</u> of the disease is reached some 20 - 45 days after the commencement of feeding the Torula-yeast diet. By this time, the necrosis is visible on examination of the liver, and light microscopy of livers sampled during the terminal stage, showed a progressive necrosis originating from the central portions of the lobes. A ramifying pattern observed by light microscopy, appeared to correspond to the branching of efferent veins, while a narrow layer of undamaged cells surrounded the portal veins. As the necrosis increased, this zone was progressively

narrowed. Schwarz (1958) suggested that the better-nourished portal cells suffered less than the more peripheral cells of the liver.

In addition to frank necrosis, a second outstanding feature of dietary necrotic liver degeneration is the presence of haemorragic areas which early workers had grouped together as "haemorrage and necrosis". Careful examination by Schwarz (1958) showed that these changes did not constitute true haemorrage but they were an "accumulation of blood within the liver sinusoids". The pattern suggested a block in the efferent vascular system possibly resulting in back-flow of blood from efferent vessels into the necrotic areas. In a study of the histological changes seen in dietary liver necrosis, Fite (1954) described a diffuse change in the nucleus of hepatocytes characterised by karyolysis and karyorrhexis - fragmentation of cell nuclei into chromatin particles. This change was consistently observed during the terminal phase of the disease. Fite (1954) also reported the existence of granules, distributed evenly in the cytoplasm, which had appeared to undergo some calcification during the latent phase of the disease. These "degenerative microbodies" were eosinophilic and were thought to derive from degenerative mitochondria. The duration of the dramatic terminal phase is only a few hours and is always followed by death of the animal.

2. <u>Selenium and chick nutrition</u>.

One aspect of the involvement of selenium in chick nutrition has already been discussed above (Chapter 3); however, the situation is considerably more complex in that several deficiency diseases involving vitamin E occur in the chick and the effectiveness of selenium in their prevention will depend on the vitamin E status of the bird. In addition, selenium and vitamin E interact with other nutrients notably sulphur amino-acids, polyunsaturated fatty acids and dietary synthetic anti-oxidants. Thus, encephalomalacia in chicks (- a lesion, in brain

tissue caused by a diminished supply of blood) is prevented by the administration of vitamin E and synthetic antioxidants and is exacerbated by linoleic acid (Dam et al 1958). Other workers (Calvert et al 1962) have reported that selenium has some effect in delaying the onset of the disease, but cannot prevent it. In addition, selenium spares the amount of vitamin E required to prevent nutritional muscular dystrophy in chicks, (Scott 1962) but will not prevent dystrophy when added to diets severely deficient in both vitamin E and sulphur aminoacids.

Selenium itself has been shown to be an essential micronutrient for the chick (Thompson & Scott 1970) independent of its inter-relationship with other nutrients. Day-old, selenium-depleted chicks were given a diet containing crystalline aminoacids and other highly purified essential nutrients including polyunsaturated fatty acids and high levels of vitamin E. The most significant abnormalities found by Thompson & Scott(1970) during selenium deficiency in the chick were poor growth and feathering (alopecia) and fibrotic degeneration of the pancreas. In addition, there was in selenium-deficient chicks, a consistent fall in the blood level of vitamin E, even when these chicks were given large amounts of vitamin E in the diet. Experiments with radioactively-labeled DL -α- tocopherol showed that the low blood vitamin E levels were a consequence of impaired absorption of fats, and large amounts of unhydrolyzed triglycerides were found in the faeces (Thompson & Scott, 1970). Blood tocopherol levels were correlated with plasma turbidity which suggests that vitamin E is normally absorbed as a constituent of lipid micelles, at least until arrival at the mucosal cells, where ester forms are presumably hydrolysed (Puddlekiewicz and Mary, 1969). The concept that lipid is a carrier for tocopherol is compatible with the results of earlier studies (Wiss et al, 1962) on the absorption of vitamin E in tissues. In addition, this study explained the changes noted by Thompson and Scott in selenium-deficient

chicks, and the low blood tocopherol levels found in children with celiac disease, cystic fibrosis, obstructive jaundice and diarrhoea (De Oliviera 1949; Fiteret al 1951; Nitowsky et al 1962).

The abnormalities in fat digestion are thought (Scott 1973) to be due to changes in the pancreas - a primary effect of selenium deficiency in the chick. Pancreatic degeneration could lead to poor fat absorption which in turn would impair the absorption of vitamin E. It appears that the decrease in pancreatic lipase, when selenium was absent from the diet, caused a failure of fat digestion and bile flow, which resulted in a failure of the proper micelle formation necessary for vitamin E absorption.

Supplementation of the diet with free fatty acids, monoglycerides and bile salts gave an improvement in vitamin E absorption by the selenium-deficient chicks, since pancreatic lipase was no longer needed for fat digestion. However, these supplements did not prevent the pancreatic changes. The selenium requirement to prevent pancreatic degeneration was dependent on the level of dietary vitamin E (Thompson & Scott 1970); at high levels of vitamin E (100 mg / kg of diet), only 0.001 mg / kg Se was required. However, at a normal vitamin E level of 10 mg / kg of diet, 0.02 mg Se / kg was needed. If monoglycerides and bile salts were not administered to the selenium-deficient chicks (i.e. if vitamin E absorption was not enhanced), the animals eventually became depleted of vitamin E. Since exudative diathesis occurs after the blood tocopherol levels have fallen to low values, this disease appears to be a consequence of vitamin E deficiency. However, this cannot be the sole cause as the disease will not occur in chicks given selenium even when they are rigorously depleted of vitamin E (Thompson & Scott 1969). The true nature of exudative diathesis, like liver necrosis in the rat, is thus an enigma. As chicks severely deficient in selenium are unable to absorb vitamin E, they automatically become depleted in both nutrients and often die after developing exudates.

However, when tissue levels of tocopherol are maintained in selenium-deficient chicks, e.g. by supplying dietary hydrolyzed fats and high levels of vitamin E, then exudates and mortality are prevented and the birds continue to develop the more specific lesions of selenium deficiency, such as the degenerative changes in the pancreas, to an advanced stage.

Thus, in the chick only pancreatic fibrosis, exudative diathesis and alopecia can be said to be truly selenium responsive while exudative diathesis is, in addition, vitamin E responsive; muscular dystrophy can be prevented by the administration of vitamin E provided that sufficient levels of sulphur-amino acids are supplied in the diet.

In general, therefore, selenium can be said to spare vitamin E for chick nutrition in three ways:

(1) Selenium preserves the integrity of the pancreas which allows, through enhanced fat digestion, proper absorption of vitamin E.

(2) Selenium aids the retention of vitamin E in blood plasma of chicks given diets low in sulphur amino acids which produce muscular dystrophy (Desai and Scott 1964, 1965).

(3) Selenium may decrease the chance of oxidative damage from peroxides via its function in glutathione peroxidase (see Chapter 9).

3. <u>Selenium and Turkey nutrition</u>:

Exudative diathesis in turkey poults was described by Creech <u>et al</u> (1957), but its pathology and etiology have not been as well researched as the disease in chicks. Symptoms in the two species are similar except that the anaemia in turkey poults was found to be macrocytic, rather than the microcytic type described by Scott <u>et al</u> (1955) in chicks suffering from exudative diathesis. Further, no changes in the protein components of blood serum (determined electrophoretically) were found in turkey poults suffering from exudative diathesis. However, selenium-depleted poults had

reduced serum albumin to serum globulin ratios, which suggests a similarity to the disease in chicks; these changes were reversed by giving α-tocophenol or brewer's yeast (a source of selenium).

Although young poults have been reported to suffer from exudative diathesis, the chief symptoms of selenium deficiency in turkey poults are myopathies of the gizzard and the heart (Scott et al 1967; Walter and Jensen, 1963). Early reports of gizzard myopathy in turkeys were described by Jungherr and Papenheimer (1937) as the only sign of vitamin E deficiency. Since then, however, several groups of workers have shown that under appropriate conditions, vitamin E-deficient poults also display nutritional muscular dystrophy (myopathy of the skeletal muscles), as well as severe myopathies of the smooth muscle (gizzard) and the cardiac muscle. Ferguson et al (1964) reported nutritional myopathy of skeletal muscle in turkey poults receiving diets containing 10% ethyl linoleate in which the methionine and arginine levels were varied. Walter and Jensen (1963) reported myopathies of skeletal, gizzard and heart muscles in young poults given a vitamin E-deficient diet, based on Torula yeast, that was also deficient in selenium. Addition of sulphur-containing amino acids to the diet was ineffective in producing any improvement; vitamin E was somewhat effective whereas selenium as sodium selenite was completely effective in preventing all symptoms.

Interest in the selenium-responsive disease of poults was revived in 1964 when several commercial flocks of young turkeys in south western Ohio were found to grow poorly and suffer a high mortality rate, with no overt signs of disease or nutritional deficiency, except for a severe hyaline degeneration of the gizzard muscle (Bruins et al 1966). Investigation revealed that the corn and soybean meal used in the turkey diets were grown on soils found by Muth and Allaway (1963) to be very low in selenium. The forages produced were thus very deficient in this element. Scott et al (1967) investigating this disease under laboratory conditions,

used a low selenium-content, semipurified basal diet containing Torula yeast, sucrose and soybean meal and protein to produce selenium deficiency in turkey poults. They reported poor growth, high mortality and myopathies of the heart and gizzard in young poults which were given the basal diet without vitamin E or methionine supplementation. Addition of either vitamin E (11 I.U. per kg of diet) or methionine (0.1% w/w) improved the growth of the poults but gizzard myopathy was not prevented and maximal growth was not achieved until 0.1 ppm Se as sodium selenite was included in the diet. The precise requirement for selenium appeared to be related to the vitamin E or methionine content of the diet: thus, in the presence of supplemental vitamin E, the selenium requirement was 0.18 ppm, absence of vitamin E from the diet resulted in an increase in the requirement to 0.28 ppm. No myopathy of the pectoral muscles was noted grossly in the deficient birds although histological exmination revealed some degeneration. Scott et al (1967) concluded that, in general, the selenium-responsive diseases of the young poult appear to develop in a certain order of prominence:

i) myopathy of the smooth muscle (gizzard)

ii) myopathy of the myocardium

iii) myopathy of the skeletal muscle

Selenium appeared to be the nutrient of primary importance for the prevention of these disorders: vitamin E was less effective while sulphur amino acids were only partially effective.

4. Selenium deficiency in lambs and calves

A myopathy in young lambs, generally referred to in veterinary circles as "stiff muscle disease", was first recognised and described by Metzger and Hagen in 1927. The disease was experimentally produced by feeding pregnant ewes herbages and/or pulses grown on selenium deficient soils. Following the identification of selenium as the curative agent for liver necrosis in the rat and for exudative diathesis in the chick,

Procter et al (1958) and Muth and co-workers (1958) showed that supplementation of the diet of the ewes with 0.1 to 1.0 ppm of selenium resulted in complete abolition of the disease in their offspring, provided that a low level of polyunsaturated fatty acid was given.

Muth and his associates at Oregon State University (1955; 1963) studied extensively the etiology and pathology of the disease which occurs in young calves or lambs born to dams fed on a diet extremely low in selenium. The name "white muscle disease" has been applied to this selenium and vitamin E-deficiency disease because of the light colour of the skeletal and myocardial muscles. The affected lambs are either born dead or may die suddenly after exertion. Post-mortem examination usually reveals clear fluid and fibrin strands in the body cavities and a congested liver (Scott, 1973). The right ventricle is usually affected by a grayish white discoloration of the myocardium, although other cavities may also be involved. Initially the lesion consists of large areas of non-inflammatory coagulative myonecrosis. This is superceded by lysis or calcification of the affected fibres and replacement by macrophages and fibroblasts.

Two further selenium-responsive diseases of sheep and cattle have been reported in New Zealand by Andrews et al (1968). Embryonic mortality in ewes, which is of considerable economic importance, has occurred in the South Island of New Zealand for decades and its incidence is often as high as 75%. These workers found that administration of selenium to the ewe just before mating, prevents this infertility. It should, however, be noted that cattle in the same areas appeared to be unaffected by the selenium-deficiency.

The second condition is selenium-responsive unthriftiness which is probably the most widespread and economically important of all the selenium-responsive diseases of New Zealand livestock. Unthriftiness is

characterised by a subclinical inability to maintain optimal growth rate. Lambs may apparently thrive for several months and then show abruptly reduced weight gains. Others stop eating, stop growing, lose weight, become dejected and die. This condition has been demonstrated in sheep and in beef and dairy cattle, and has been shown to be completely responsive to selenium administration.

5. Selenium Deficiency in pigs

When weanling pigs are given diets deficient in both vitamin E and selenium, necrotic liver degeneration (hepatosis dietetica) occurs; this usually results in death unless the diet is supplemented with either vitamin E or selenium (Eggert et al, 1957). Other workers (Pellegrini, 1958) have reported both liver and cardiac myopathy (mulberry heart), as well as skeletal muscle degeneration in pigs fed a vitamin E-deficient diet without added selenium. These deficiency diseases are prevented by supplementing the diet with either vitamin E or selenium but not cysteine. Orstadius et al (1963) found that pigs receiving a diet deficient in both selenium and vitamin E showed elevated glutamic oxaloacetic transaminase (GOT) levels in the plasma, which were reduced by supplementing the diet with either of the nutrients.

CHAPTER 5

BIOCHEMICAL ACTION OF SELENIUM

A. Respiratory failure of rat liver:

The inability of liver slices and homogenates obtained from rats in the latent phase of dietary necrotic liver degeneration, to maintain normal respiration *in vitro* has been discussed in the last chapter. The possible mechanism of the biochemical action of selenium will be considered here, in order to explore the cause of these metabolic lesions in the absence of dietary selenium. Chernik *et al* (1955) showed that liver slices derived from rats maintained on a vitamin E-supplemented diet (50mg % DL-α-tocopheryl acetate) were able to maintain a linear rate of oxygen uptake for over two hours while slices from livers of rats in the latent phase consumed oxygen at a normal rate for thirty minutes and thereafter, the rate of respiration declined until it was only 30% of normal after 90 to 120 minutes of incubation. Although livers that were histogically normal also showed a decline in respiratory activity, there appeared to be some relationship between the period that the rat was given the selenium - and vitamin E-deficient Torula yeast diet, and the severity of the respiratory lesion. Thus, rats given the basal diet for 21-29 days showed a 57% decline in respiration while in those rats maintained on the diet for 50-70 days, the rate of respiration was 29% of that recorded for rats on

normal diets.

A comparison of the oxygen consumption in kidney and diaphragm of rats given the deficient and supplemented diets showed that, in contrast with liver, normal respiration was maintained in other tissues during the latent phase.

Chernik et al (1955) further showed that supplementation of the basal diet with vitamin E, cystine or selenium (added as Factor 3) prevented the development of the metabolic defects that produced respiratory decline. These substances were also effective in preventing dietary necrotic liver degeneration which is inevitable in rats fed the basal diet alone and which usually occurs several weeks after the onset of respiratory failure. These results suggested to the authors that there existed a definite relationship between the earlier metabolic changes and the degenerative necrosis that followed.

In addition to dietary necrotic liver degeneration, other forms of hepatic injury were tested (Chernik et al, 1955) for the defect in oxygen consumption. Neither fatty nor necrotic livers from other causes were found to exhibit respiratory decline; however, certain drugs, such as naphthoquinones (Ball et al, 1947) and pamaquine (Elderfield et al, 1949) are known to induce similar effects when incubated with liver slices. The inhibitory action of these drugs on respiration in liver may be due to their slow rate of

penetration into the cells (Ball et al, 1947) or to their conversion into inhibitory degradation products (Elderfield et al, 1949) or to the uncoupling of oxidative phosphorylation (Lardy and Elvehjem, 1945). As the first two cases do not seem to apply to the metabolism of prenecrotic liver in dietary necrotic liver degeneration, Chernik et al (1955) suggested that "failure in oxidative phosphorylation may be involved in the progressive decline in respiration."

When α-tocopherol was administered in the diet of selenium-deficient animals, Chernik et al (1955) found that respiratory failure was prevented and necrotic liver degeneration was arrested. Because of the anti-oxidant properties of α-tocopherol, the activity of thirteen synthetic antioxidants was tested in rats given the necrogenic diet, with regard to their ability to prevent both necrosis (Schwarz, 1958) and respiratory decline (Mertz and Schwarz, 1959). It was found that while water-soluble antioxidants, such as ascorbic acid and methylene blue, were only partially protective against necrotic degeneration, lipid-soluble anti-oxidants, such as ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) and DPPD (N,N^1-diphenyl-p-phenylene diamine) were highly effective; others, such as n-propyl gallate, were ineffective. Further study of the effect of these antioxidants in vitro (Mertz and Schwarz, 1959) showed that most had some protective activity in preventing respiratory decline when added

at catalytic dose levels, and the potency *in vitro* closely followed the activity *in vivo*. Methylene blue was however, an exception and was found to be highly active *in vitro* while on addition *in vivo*, it was only partially effective; DPPD was found to be highly active in both systems and closely resembled the behaviour of α-tocopherol.

Rodnan *et al* (1956) further described a series of experiments in which the effect of the intraportal injection of various tocopherols on the respiratory defect in liver slices was investigated; the rate of respiration in liver slices was determined both before and after administration of the tocopherols by this means. It was found that although emulsions of the various tocopherols and water soluble D-α-tocopheryl polyethylene glycol-1000 succinate did not markedly alter the initial rate of oxygen consumption, respiratory decline was reversed and the degree of decline of O_2-consumption diminished with increasing doses of tocopherol. For male, weanling rats (18-22 days old) a dose of 0.19mg of emulsified DL-α-tocopherol was required for 50% reversal of the respiratory lesion, and physiological amounts of α-tocopherol were, in general, found to be completely protective. Further, β-, γ- and δ-tocopherols were all found to exert some measure of protection although to varying degrees: γ-tocopherol was twice as active as β- and five times as active as δ-tocopherol; sodium selenite was

found to be completely ineffective. It would thus appear that whereas the tocopherols were able to be incorporated rapidly into their site of operation, selenium needs further conversion perhaps to a metabolite or incorporation into an active lipid or protein before it can become active.

When rat liver homogenates were used to study respiratory decline, it was found (Corwin and Schwarz, 1960a, 1960b) that the rate of O_2-uptake by liver homogenates of rats given the necrogenic diet, decreased when α-ketoglutarate or succinate were used as substrate. The administration of α-tocopherol and DPPD, whether added <u>in vivo</u> to the diet of the rats or <u>in vitro</u> to the incubation mixture, led to complete prevention of the respiratory decline when α-ketoglutarate was used as substrate. With succinate as substrate, larger quantities of tocopherol and DPPD were required, and protection was incomplete.

In experiments with isolated mitochondria, Corwin and Schwarz (1959) showed that the behaviour of liver mitochondria from prenecrotic rats was similar to that of normal rats. However, when NAD was also added to the medium, a decline in the oxidation rate of succinate was obtained (Corwin and Schwarz, 1959) and this was thought to be due to product inhibition of succinate oxidation caused by the accumulation of oxaloacetate. The addition of α-tocopherol reversed this decline but dietary selenium was without effect.

Corwin (1961), upon further investigation, suggested the involvement of a microsomal factor since a decline in the rate of oxidation could be demonstrated in mitochondria from deficient rats when microsomal and mitochondrial fractions were combined and when α-ketoglutarate was used as substrate. In early studies of the intermediary metabolism of prenecrotic livers, attention was focused on the lipoyldehydrogenase portion of the α-ketoglutarate dehydrogenase enzyme complex in the tricarboxylic acid cycle. Schwarz et al (1962) demonstrated a close relationship between lipoyl dehydrogenase and respiratory decline in homogenates, and they suggested that selenium and vitamin E were involved in the α-ketoglutarate dehydrogenase enzyme complex; however, when the complex was analysed for selenium, it was found (Schwarz, 1962) that the element was not present in stoichiometric amounts.

More recent work by Bull and Oldfield (1967) has diverted attention to the enzyme pyruvate dehydrogenase, which has a mechanism of action similar to that of α-ketoglutarate dehydrogenase. Rats were given a diet deficient in both selenium and vitamin E for 5 to 11 weeks and necrosis was prevented by the administration of ethoxyquin. Bull and Oldfield (1967) demonstrated a defect in pyruvate oxidation which was corrected by dietary selenium and vitamin E. This study confirmed the findings of Schwarz et al (1962) in uncovering a defect in enzyme systems of rats given

selenium - and vitamin E-deficient diets, and also showed that although adequate amounts of the antioxidant ethoxyquin were administered to prevent lipid peroxidation, the onset of the biochemical lesion of respiratory decline was not inhibited. This confirmed the observation of Corwin (1962) that there was no correlation between the concentration of thiobarbituric acid reactive material, taken as a measure of lipid peroxidation, and the extent of respiratory decline.

The NAD-dependent decline in succinate oxidation by prenecrotic rat liver mitochondria has been studied more extensively by Johnson and co-workers at the Ohio State University, Columbus, Ohio. In a study of a range of cofactors and substrates (Grove et al, 1965; 1966), NAD was found to be the only cofactor that was required to demonstrate respiratory decline in liver mitochondria isolated from rats given a vitamin E-deficient Torula yeast diet with selenium supplementation. Grove and Johnson (1967) suggested that NAD was less efficiently utilized in vitamin E-deficient mitochondria and a subsequent report (Yeh and Johnson, 1972) indicated that the permeability of mitochondria to NADH was increased in vitamine E-deficiency, implying a possible change in the intactness of the mitochondrial membrane. In studies of membrane integrity in mitochondria from vitamin E-deficient rats, (Yeh and Johnson, 1973), the effects of aging, osmotic shock and digitonin treatment were

investigated; it was found that liver mitochondria from deficient animals showed a rapid loss of respiratory control as compared to supplemented animals, which indicated that deficient mitochondria have a less compact structural organization, since respiratory control would be affected by a change in the permeability or intactness of the mitochondrial membrane. In addition, experiments on NADH oxidation showed that oxidation was increased in vitamin E deficiency. Since the mitochondrial oxidation rate of a substrate depends upon the rate of substrate penetration (Haslam and Krebs, 1968), Yeh and Johnson (1973) proposed that the increased rate of oxidation of NADH in deficient mitochondria was due to the increased penetration of NADH.

In an investigation of the involvement of cytoplasmic factors in respiratory decline, Yeh and Johnson (1975) showed that the addition of liver cytoplasm from either vitamin E-deficient or normal rats arrested the decline in mitochondrial respiration by vitamin E-deficient rats, and that the cytoplasmic factors responsible were enzymes of the malate shuttle and of transamination, plus dialysable metabolites associated with these enzymes in NAD-generation in mitochondria.

Thus, a possible explanation for the occurence of the lesion known as respiratory decline, has been proposed which is satisfactory in terms of present-day knowledge of mitochondrial metabolism. However, it is

important to consider the observed structural effects of vitamin E on the mitochondrial membrane and the nature of the relationship of selenium to respiratory decline.

B. The possible role of glutathione peroxidase and vitamin E in respiratory decline

The physiological functions of glutathione peroxidase and its mechanism of action are described in greater detail in Chapter 9. The possible involvement of the enzyme in the biochemical lesion of respiratory decline is discussed in this section.

Glutathione peroxidase was first described by Mills in 1957, as an enzyme in bovine erythrocytes which catalysed the reduction of hydrogen peroxide with GSH as the donor of reducing equivalents. Studies on the effects of dietary selenium and vitamin E (severally and together) on haemolysis and oxidative damage to rat erythrocytes *in vitro*, led to the discovery by workers at the University of Wisconsin, that glutathione peroxidase is a selenoenzyme (Rotruck *et al*, 1973). It had been demonstrated (Rotruck *et al*, 1971) that, in common with vitamin E which was known to protect erythrocytes from haemolysis (Rose and Gyorgy, 1950), dietary selenium also had a protective effect when glucose was included in the incubation medium; the effect of glucose was limited to erythrocytes from rats receiving adequate selenium however, and was not

apparent when selenium-deficient rats were tested. Protection against haemolysis by dietary vitamin E did not require the addition of glucose to the incubation medium, suggesting a different mode of action from selenium. Cohen and Hochstein (1963) observed that glucose not only protected erythrocytes from H_2O_2-induced haemolysis, but also prevented the hydrogen peroxide-induced oxidation of haemoglobin to methaemoglobin. The effect of glucose was thought (Rotruck et al, 1972) to be mediated through its ability to maintain high levels of reduced glutathione (GSH) by providing a supply of reducing equivalents via the pentose phosphate pathway (see Fig. 9-8), for the reduction of GSSG to GSH.

The concept that the role of selenium was not in the formation of GSH, but in its utilization was tested by Rotruck et al (1973); the ability of GSH, when added to an erythrocyte haemolysate, to protect against haemoglobin oxidation by ascorbate or H_2O_2 was investigated in the absence or presence of dietary selenium. Only in the rats given selenium did added GSH protect the haemoglobin. Rotruck et al (1973) further showed that GSH utilisation was more rapid in selenium-fed rats than in deficient animals and that inhibition of GSH removal in the latter group was due to the almost complete absence of the enzyme glutathione peroxidase. Results of preliminary experiments in which $Na_2\,^{75}SeO_3$ was administered to rats revealed that most of the ^{75}Se of erythrocytes accompanied the glutathione peroxidase

activity during purification on DEAE-Sephadex columns; this, then, was the first indication obtained by Rotruck et al (1973) and subsequently confirmed in other experiments, that selenium is an integral part of glutathione peroxidase.

The effects of vitamin E and selenium on liver mitochondrial function and the metabolic consequences in liver of dietary deprivation of these two nutrients have been discussed above. The possible role of glutathione peroxodase in the biochemistry of these metabolic lesions must now be considered, together with the biological significance of peroxidative phenomena.

The antioxidant hypothesis was proposed by Tappel (1962) to explain the close relationship between selenium and vitamin E and their interaction with other nutrients. This hypothesis is discussed in greater detail in Chapter 6 of this thesis and only the major points will be mentioned here. Early observations by Davies and Moore (1941) about the nutritional interaction of vitamin A and vitamin E, led to the suggestion that the function of vitamin E was to protect vitamin A from oxidation in vivo. During the years that followed this observation, dietary fat, particularly unsaturated fat, was found (Harris and Embree, 1963) to have an exacerbatory effect on vitamin E-deficiency disease and certain synthetic antioxidants were shown to be protective against

vitamin E-deficiency disease (Dam, 1957). Hickman (1949) proposed that vitamin E might be considered to protect both structural elements and intracellular metabolites against oxidative damage and Dam (1949) discussed the possible interaction between vitamin E and tissue lipid peroxides. Tappel (1953) then combined the many diverse observations, on the function of vitamin E, in a hypothesis which explained all the manifestations of vitamin E deficiency disease in terms of one function of α-tocopherol, namely its ability to inhibit the peroxidation of polyunsaturated fatty acids *in vivo*.

The biological antioxidant theory has been critically and thoroughly reviewed by Green and Bunyan (1969) and they concluded that this explanation for the mode of action of vitamin E and selenium could not be regarded as viable. The major points of evidence which could not be answered by the theory and which were taken to argue against it are discussed in Chapter 6.

Since the publication in 1969 of the review by Green and Bunyan, the view that the function of vitamin E is closely associated with membrane structure has gained wide acceptance; in particular, the hypothesis of Diplock and Lucy (1973) that there may be a physicochemical interaction between the phytyl side chain of α-tocopherol and the fatty acyl chains of polyunsaturated fatty acids is now regarded as a more comprehensive alternative to the antioxidant hypothesis

of Tappel. Another major discovery since 1969 and one which has a direct bearing on the criticisms by Green and Bunyan, is the observation that glutathione peroxidase is a selenoenzyme.

Consideration of the phenomenon of respiratory decline must therefore involve the selenium dependence of glutathione peroxidase, the ability of α-tocopherol to function as an antioxidant and the possible involvement of vitamin E in membrane physiology. It is evident that respiratory decline is primarily a mitochondrial lesion; Green and O'Brien (1970) in studies of the cellular localisation of glutathione peroxidase, have demonstrated that the enzyme is present both in the cell soluble fraction and within the mitochondria. Thus, lipid hydroperoxides formed within the outer mitochondrial membrane would be destroyed by cytosolic glutathione peroxidase while hydroperoxides formed within the inner membrane would be attacked by the enzyme located in the intramitochondrial fluid. Of particular interest also, are the experiments of Grove et al (1965; 1966; 1967) on the phenomenon of respiratory decline and their conclusion (Yeh and Johnson, 1973) that the lesion is entirely caused by alterations in the integrity of the mitochondrial membrane. It is likely, therefore, that in the prenecrotic liver, diminishing supplies of α-tocopherol and selenium lead to a progressive failure of inhibition of peroxidation; either nutrient

can prevent this process: selenium, through its action in glutathione peroxidase, removes the products of peroxidation, and vitamin E, by its function in maintaining membrane structure or via its action as an antioxidant, inhibits peroxidation. A change in the intactness of the membrane would lead to increased rate of penetration of NADH into the mitochondria, as observed by Yeh and Johnson (1973), and thus to the phenomenon known as respiratory decline.

CHAPTER 6

INTERACTION OF SELENIUM WITH VITAMIN E

A. Discovery of vitamin E

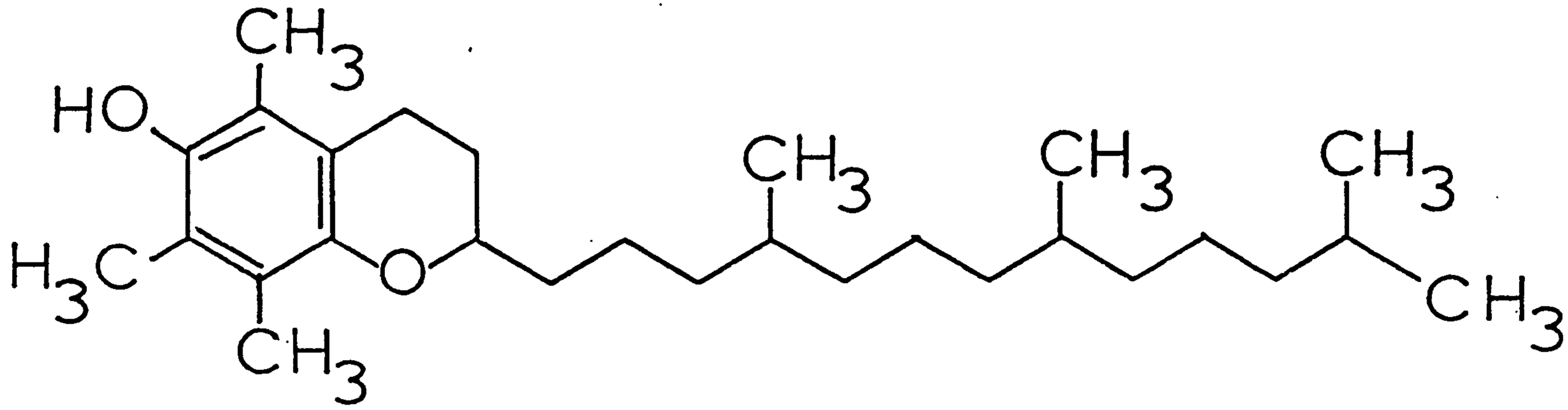
Vitamin E was discovered in 1927 by Evans and Burr, who described a lipid-soluble dietary factor which they called "vitamine E", that was capable of preventing reproductive failure in rats. Reproductive abnormalities in rats given special diets were first noted by Mattill and Conklin (1920); they found that, although the semi-purified diets provided adequate levels of vitamins A, B, C and D, reproduction was not achieved. In 1922, Long and Evans published a monograph on the oestrous cycle of the rat and the effects of dietary changes on its duration were later recorded by Evans and Bishop (1923). Further, Evans and Bishop (1922) carried out a series of experiments on sterility in female rats and found that rats given a diet containing rancid lard, became sterile. The ovarian function of these animals did not differ much from that of normal rats, but the disease was characterized by placental malfunction which resulted in foetal death and resorption. Evans and Bishop demonstrated that natural foodstuffs contained a factor "X" which prevented or cured the disorder produced by the purified diet. It was found that wheat germ, fresh lettuce and even dried alfalfa leaves contained the necessary factor "X" to restore fertility to previously sterile animals; on the other hand, the

administration of cod-liver oil, known to be rich in vitamins A and D, was ineffective against the disease.

The experiments of Evans and Burr (1927) showed that sterility in male rats was permanent and incurable: the male germ-cells degenerate and cannot be reformed. However, later work by Harris and Mason (1956) showed that, in the early stages of the disease, degeneration is reversible by the administration of the necessary cofactors. In more recent work with hamsters, Mason and Mauer (1975) showed that daily oral supplements of 2mg D-α-tocopheryl acetate were marginally effective, whereas doses of 10mg were sufficient to prevent damage to the germinal epithelium, and effect the reappearance of spermatozoa in the epididymal duct and the removal of acid-fast pigment.

In addition to the manifestations of deficiency mentioned above, there were early signs that vitamin E might be more than just an antisterility vitamin. Although synthesized by plants, vitamin E (Fig. 6-1) is known to be essential for all species of animals studied and is necessary for the normal metabolic processes of many different tissues. Dietary deficiency of the vitamin leads to a great variety of disease symptoms in different species of animals and several chemically unrelated compounds have been found to be capable of reversing some vitamin E deficiency symptoms; these compounds include synthetic antioxidants, (Dam, 1953) coenzyme Q, selenium and sulphur amino acids

Fig. 6-1 α-Tocopherol



(Schwarz, 1951). In addition, the experiments of Mattill (1927) and Mattill and Golumbic (1942) demonstrated a nutritional antagonism between vitamin E and dietary polyunsaturated fatty acids (PUFA). To these important observations must now be added the recent discovery that selenium is an integral functional part of glutathione peroxidase (Rotruck et al, 1973), an enzyme which probably functions in vivo to destroy lipid peroxides (see Chapter 9).

There are two basic concepts of the biological role of vitamin E. One is that vitamin E functions in the animal as a lipid antioxidant and free-radical scavenger, the other being that vitamin E plays a structural role in biological membranes.

B. Selenium, Vitamin E and Tissue Peroxidation

1. The Antioxidant Hypothesis

Due to the recognition of the nutritional interrelationship between dietary PUFA and vitamin E (Mattill and Golumbic, 1942) and the discovery that some unphysiological antioxidants had vitamin E activity (Dam, 1953), an attempt was made by Horwitt (1961) and more coherently by Tappel (1962) to formulate a hypothesis to explain the function of vitamin E and its interaction with other nutrients. The hypothesis supposed that the biological function of α-tocopherol was solely that of a lipid antioxidant and free-radical scavenger, and that the varied spectrum of vitamin E-deficiency

diseases was a consequence of non-specific tissue damage by free-radical species produced as a result of lipid peroxidation *in vivo*.

2. Origins of the antioxidant theory

Although the antioxidant properties of α-tocopherol were known before its synthesis by Karrer *et al* in 1938, the attribution of its biological function to those properties did not follow for several years. Davies and Moore (1941) suggested that a function of vitamin E *in vivo* might be to suppress the rate of depletion of vitamin A in liver. Hickman *et al* (1942) showed that vitamin E increased the utilization of vitamin A for growth, and suggested that α-tocopherol might function to protect all labile substances from oxidation *in vivo* and Hickman (1949) later proposed that vitamin E acted generally throughout the animal organism as a preservative agent for both metabolites and structural elements. Dam (1949) discussed the possible relation between vitamin E deficiency and tissue lipid peroxides and Tappel (1953) first combined all the different theories and opinions on the mode of action of vitamin E in a comprehensive theory to account for manifestations of vitamin E deficiency diseases.

In a critical and thorough review of the biological antioxidant theory, Green and Bunyan (1969) suggested two fundamental premises, potentially susceptible to experimental testing, which must be upheld

if the theory is to be acceptable. These were:

a) If vitamin E deficiency diseases are caused by peroxidation *in vivo* of unsaturated fatty acids, it should be possible to demonstrate the existence of these peroxides in tissues. Further, their amount should increase as vitamin E deficiency proceeds i.e. as the tissue vitamin E level decreases, and as the level of dietary PUFA increased. Conversely, administration of vitamin E should lead to a decrease in tissue peroxide level.

b) Vitamin E deficiency should result in increased damage to oxidasable components of intracellular structures and substrates e.g. lysosomes and α-tocopherol itself; the destruction should be exacerbated by increased dietary PUFA levels.

3. Evidence for Lipid Peroxidation

The test most frequently used to measure lipid peroxides is the estimation of malonyldialdehyde (MDA) by the thiobarbituric acid reaction (Ottolenghi, 1959). MDA is formed *in vitro* as a breakdown product during the oxidation of certain polyunsaturated acids such as linolenic and arachidonic acid, but not linoleic acid (Dahle *et al*, 1962); the amount of MDA produced is thus quantitatively related to the extent of peroxidation. Estimates of the MDA content of homogenates, prepared

from tissues of vitamin E-deficient animals, vary widely, depending upon the care taken during homogenization and preparation in order to exclude the possibility of oxidation of the polyunsaturated fatty acids during handling. If the precautions to exclude oxygen are good, MDA values are usually very low and cannot be correlated with the development of vitamin E-deficiency disease. Thus, Bunyan et al (1962) found no relation between MDA values and the development of exudative diathesis (a vitamin E- and selenium-responsive disease) in the chick, and Bunyan et al (1963) found no correlation between liver MDA values and the onset of nutritional necrosis in the rat.

The direct measurement of lipid peroxides in tissues is very difficult. Sensitive spectrophotometric techniques are required to measure the minute amounts of iodine released by the peroxide upon addition of iodide, and great care must be taken to prevent peroxidation of the tissue lipids during handling. Early workers (Dam and Granados, 1945; Christensen et al, 1958) reported high peroxide values (up to 190 μequiv per gram) in tissues of vitamin E-deficient rats; however, this was almost certainly due to their inability to exclude peroxidation in vitro. Experiments by Bunyan et al (1967), in which special precautions were taken to exclude oxygen, resulted in much lower peroxide values (10-40 μequiv per g tissue) and, more importantly, the finding that these values were unaltered

by substantial increases in the dietary lipid unsaturation, and by deprivation of vitamin E.

4. Damage to Lysosomes

According to the hypothesis proposed by Tappel (1962), the peroxidation processes *in vivo* will catalyse the generation of free radicals that are damaging to subcellular structures and intracellular metabolites. Thus, Tappel considered that damage to lysosomes would cause release of lysosomal enzymes resulting in widespread hydrolytic damage which, Tappel suggested, would account for all the varied manifestations of vitamin E deficiency. Zalkin *et al* (1962), in studies on muscular dystrophy in the vitamin E-deficient rabbit, found increases in several hydrolases in dystrophic rabbit muscle and liberation of enzymes into the cell sap. Similarly, Desai *et al* (1964) and Bunyan *et al* (1967a) found increased hydrolase activity in the breast muscle of chicks with muscular dystrophy. However, Desai *et al* (1964a), in a detailed time-sequence study of muscular dystrophy in the chick, found that dietary methionine could reverse the lesions even though, in the absence of dietary vitamin E, the tissues remained highly peroxidisable. Bunyan *et al* (1967b) found increases in the activities of some, but not all, lysosomal enzymes in the uterine contents of vitamin E-deficient rats during foetal resorption, compared to normal rats during gestation. Similarly, Bunyan *et al*

(1967c) found hardly any increases in lysosomal hydrolases in tissues of rats with nutritional liver necrosis, which could be associated with vitamin E and selenium deficiency. There is thus no clear evidence that increases in hydrolase activity are caused by increases in lipid peroxidation and, in muscular dystrophy in chicks, there is evidence that they are not (Desai, et al, 1964a).

5. Metabolism of Tocopherol

The antioxidant theory regards the processes in which vitamin E functions in vivo as mechanistically and kinetically similar to the antioxidant-inhibited autoxidation of an unsaturated fat, that can be observed in vitro. As Witting (1965) has stated, it is important to recognise that the inhibition of lipid peroxidation by a substance such as α-tocopherol is essentially a competitive reaction; therefore, peroxidation always proceeds at a finite rate in the presence of inhibitors and the oxidation of the fatty acid substrate is accompanied by oxidation of the inhibitor. Thus, as peroxidation is increased in vivo by the addition to the diet of more unsaturated fat, the rate of oxidative destruction of tocopherol should increase.

Early experiments, prior to 1964, had indicated that there was no difference in the rate of destruction of tocopherol, between vitamin E-deficient and normal animals (Blaxter et al, 1953; Fitch and Dinning, 1963);

the difficulty of measuring small amounts of α-tocopherol was responsible for the lack of direct experimental tests. Green et al (1967) studied the metabolism of ^{14}C-labelled α-tocopherol in conditions of vitamin E deficiency when dietary lipid stress was applied. In rats given up to 20% of highly unsaturated dietary fatty acids (derived from cod-liver oil), there was no accelerated destruction of tocopherol compared to rats given similar amounts of saturated fat. Similarly, Diplock et al (1967) found no increase in destruction of ^{14}C-tocopherol in the cerebella of chicks immediately prior to the onset of encephalomacia, or in the breast muscle and other organs prior to the development of muscular dystrophy or exudative diathesis. In a study of dietary liver necrosis in the rat, Green et al (1967a) found no evidence of increased destruction of ^{14}C-α-tocopherol in the liver immediately prior to the onset of the disease. There is therefore no evidence to support the concept that either deficiency of vitamin E or dietary fat stress is associated with an increased destruction of vitamin E.

As judged by the two criteria mentioned in section B.2 of this chapter, the antioxidant hypothesis (Tappel, 1962) in its original form appears to fail. In their review, Green and Bunyan (1969) concluded that, "although the theory is capable of uniting and explaining a large body of experimental observation, all the evidence for it is circumstantial". They went on to say:-

> "All attempts at direct proof of the theory by testing the two fundamental premises on which it is based have yielded results that, in fact, are contrary to the requirements of the theory. All direct attempts to find an increase in lipid peroxidation in the tissues of vitamin E-deficient animals have failed, and in general, the onset of disease cannot be correlated with tissue peroxidation. Secondly, the evidence all shows that the dietary stresses that are supposed to lead to increases in tissue peroxidation, whether they be produced by increased unsaturated fat or by toxic agents, in fact do not result in increased catabolism of α-tocopherol, secondary antioxidants such as ascorbic acid, or sulphydryl compounds, vitamin A or other so-called peroxidisable intracellular substances."

Since the publication of the review by Green and Bunyan (1969) another function of vitamin E in biological membrane structure has been proposed (Diplock and Lucy, 1973) and the role of selenium in glutathione peroxidase has been discovered (Rotruck et al, 1973).

C. The Effect of Vitamin E on the oxidation state of selenium

As it is known that a close relationship exists between the metabolic functions of selenium and vitamin E, Diplock et al (1968) investigated the possibility that the oxidation-reduction properties of α-tocopherol might be directed towards selenium. Two questions had arisen which were subjected to experimental investigation:

a) Was it possible to detect different valence states of selenium in animal tissues, and was the reduction of selenium accomplished by the animal organism?

b) Did vitamin E have any effect on the oxidation state of selenium, and was the reduction of selenium aided by the presence of the vitamin?

When ^{75}Se as sodium ^{75}Se-selenite was administered to rats under different dietary conditions (Diplock et al, 1971), the appearance of $^{75}SeO_3^{2-}$ in liver subcellular fractions and its oxidation or reduction were studied. The diets used were either vitamin E deficient or supplemented with the vitamin, and rats were pre-depleted of selenium by the administration of the selenium-deficient, Torula-yeast based diet for a short period. The distribution of ^{75}Se and its oxidation and reduction were studied in liver homogenate and in mitochondrial, microsomal and supernatant fractions. Diplock et al (1971) found that neither the amount of ^{75}Se in liver nor its distribution among the liver subcellular fractions was affected by dietary vitamin E. However, in vitamin E-supplemented rats, about 36% of the total selenium in the mitochondrial fraction and about 43% in the microsomal fraction was present as $^{75}Se^{2-}$ (selenide). In the absence of dietary vitamin E, these figures became only 25 and 30% respectively. Selenide was detected by treating the liver fraction with hydrochloric acid and passing N_2 through the mixture to drive off $H_2{}^{75}Se$ formed from any tissue selenide present. The loss of radioactivity from the fraction was attributed to tissue selenide, and this assumption was later confirmed (Diplock et al, 1973) in clear-cut experiments to

identify and differentiate hydrogen selenide and dimethyl selenide. These tests are discussed in Chapter 8 of this thesis.

Diplock et al (1971) found that, in order to detect substantial amounts of selenide and to demonstrate the difference between vitamin E-deficient and supplemented rats, it was necessary to include either β-mercaptoethanol and/or α-tocopherol in the media used for the experiments. On dialysing the fractions, mitochondrial and microsomal $^{75}Se^{2-}$ were found to be retained within the membrane, indicating that it was probably protein-bound. These results were summarized as follows:

(i) Selenium was present in tissues as $^{75}Se^{2-}$, $^{75}SeO_3^{2-}$, and a higher oxidation state.

(ii) A greater proportion of the ^{75}Se was present as $^{75}Se^{2-}$ when the animal also received vitamin E.

(iii) The $^{75}Se^{2-}$ was very susceptible to oxidation in vitro.

(iv) The microsomal and mitochondrial $^{75}Se^{2-}$ appeared to be largely protein-bound.

The possibility that post-mortem autolysis was responsible for the smaller proportion of tissue selenium in the absence of dietary vitamin E, was investigated (Diplock et al, 1971) and found to be invalid. In addition, the possible involvement of intestinal micro organisms in the conversion of SeO_3^{2-} to Se^{2-} was investigated in experiments where selenite was

administered intravenously and the micro-organisms were largely eliminated from the gastrointestinal tract by pretreatment of the rats with neomycin sulphate. The results obtained were indistinguishable from those of earlier experiments where selenite was given orally. It was thus concluded (Diplock et al, 1971) that a specific unique endogenous mechanism exists in animal tissues to effect the reduction of selenite (SeO_3^{2-}) to selenide (Se^{2-}).

Caygill et al (1971) studied the distribution of selenide in liver cells using carefully defined fractionation procedures that employed zonal centrifugation. Conditions were established so that separation of lysosomal, mitochondrial, smooth-and rough-surfaced endoplasmic reticulum fractions, and soluble fraction could be routinely achieved; marker enzyme techniques and electron microscopy were used to identify the fractions. When different dietary treatments were studied, it was found that in rats given an adequate (stock) diet, selenide was particularly associated with the mitochondrial fraction; in vitamin E-deficient rats, little selenide was found and the buoyant density of the mitochondria was increased, whereas refeeding with vitamin E showed a restoration of the normal pattern. In vitamin E- and selenium-deficient rats, no particular association of selenide with mitochondria was found.

Examination of the microsomal regions of the gradients showed that, in adequately fed rats, there was

a concentration of selenide, particularly with the smooth endoplasmic reticulum. This was not observed in vitamin E-deficient rats and re-feeding with vitamin E showed a restoration of the normal pattern, even in the absence of dietary selenium. Caygill et al (1971) therefore concluded that protein-bound selenide is a major component of rat liver subcellular organelles and that its presence depends on the presence of vitamin E in the diet. They also proposed that the selenide might form part of the active center of a class of catalytically active non-heme iron proteins. Miyake et al (1967) have demonstrated the presence of an iron-sulphur protein with sulphur present as acid-labile sulphide, in adrenal cortical mitochondria; similarly Beinert and Lee (1961) have established the existence of non-heme iron-sulphur proteins in ox-heart mitochondria. Caygill et al (1971) suggested that in the absence of dietary vitamin E, the turnover of selenide-containing proteins would be expected to accelerate; where the level of dietary selenium is adequate to satisfy the increased need for selenide, no lesion would be expected to develop. Thus, it was shown (Caygill et al, 1971) that repletion of selenium-and vitamin E-deficient rats with vitamin E alone did not result in an increase in the mitochondrial selenide formed from $^{75}SeO_3^{2-}$. It was further speculated that α-tocopherol might act in vivo to stabilize the mitochondrial membrane structure, and that the observed failure to replace mitochondrial selenide in selenium-

and vitamin E-deficient rats refed vitamin E was caused by an underlying instability of the architecture of the mitochondrial membranes.

D. Vitamin E and Membranes

The experimental work of Diplock and co-workers, which has been outlined above, indicates that an anti-oxidant function of vitamin E *in vivo* may be to protect a reduced form of protein-bound selenium from oxidation. If the antioxidant function of vitamin E is indeed this, in addition to or rather than the inhibition of the autoxidation of polyunsaturated fatty acids, as suggested by Tappel (1962) in the biological anti-oxidant theory, the question then arises as to the reason for the undoubted increase in vitamin E requirement shown when dietary levels of PUFA are increased (Mattill and Golumbic, 1942). Much circumstantial evidence exists that vitamin E may play a structural role in biological membranes, and, since PUFA are also involved as a part of the structure of membrane phospholipids, it seemed likely that the inter-relationship between the two might occur within membrane structures. Diplock and Lucy (1973) suggested that "α-tocopherol may play a physico-chemical role in the stabilization of biological membranes that contain high levels of polyunsaturated fatty acids, by virtue of lipid-lipid interactions between the vitamin and the unsaturated fatty acids." It is relevant to note that α-tocopherol

has been used as the hydrocarbon solvent for the preparation of bilayer membranes of phospholipid (Goldup et al, 1970), in which it is thought to behave as a filler, giving stability to the phospholipid membrane; it has also been shown that α-tocopherol is itself capable of forming a thin membrane separating two aqueous phases (Serfert et al, 1970). Rose and Gyorgy (1950) showed that vitamin E functions specifically to prevent the increased haemolytic susceptibility to dialuric acid or hydrogen peroxide of erythrocytes derived from vitamin E-deficient rats; more recently, Lucy and Dingle (1964) demonstrated that haemolysis by added retinol, of rabbit erythrocytes in vitro was inhibited by vitamin E. In addition, other compounds such as squalene, phytol, phylloquinone, 6-O-acetyl-α-tocopherol and ubiquinone-30 also inhibited the haemolysis, whereas classical antioxidants such as hydroquinone and DPPD had no effect. These findings indicated that the protective action of the inhibitors might be due to a steric function of their hydrocarbon side chains, and the observed interactions between retinol and vitamin E led to the suggestion (Lucy, 1966) that the liquid-like properties of the hydrocarbon side chain of α-tocopherol might play an important part in the stabilization of biological membranes in general, in a manner unrelated to the antioxidant properties of tocopherol.

Other workers have investigated biological phenomena which indicate that vitamin E may play a role

in the stabilization of biological membranes. Thus, Levander and Morris (1971) reported that dietary vitamin E, and also selenium, caused a stimulation of transport of rubidium ions from the medium, into liver slices; no correlation was found between total lipid peroxidation and transport of Rb^+ and these workers proposed that vitamin E and selenium might function to maintain transcellular cation gradients by stabilizing the plasma membrane of cells. Molenaar et al (1968) examined the ultrastructure of membranes of ultrathin sections of jejunum by electron microscopy and found that, in vitamin E deficiency, there was a marked loss of contrast of certain membranes. Thus, in two patients with abetalipoproteinemia (a disease concerning disturbance of fat and fat-soluble vitamin absorption), jejunal biopsies revealed a loss of positive contrast of membranes postfixed with osmium tetroxide. Treatment with vitamin E for four months restored the appearance of the membranes to normal. Similar phenomena were observed (Molenaar et al, 1970) in jejunal epithelial cells of vitamin E-deficient Pekin ducklings, where much of the positive contrast of mitochondrial, rough endoplasmic and outer nuclear membranes was lost. These findings were interpreted as being due to a decrease in the polyunsaturated fatty acid content of the membranes affected. Further studies on mitochondrial membranes (Vos et al, 1972) showed that the percentage of linoleic (C18 : 2ω6) and

arachidonic (C20 : 4 ω6) fatty acids in the inner mitochondrial membrane was consistently lowered in preparations from vitamin E-deficient ducklings. Thus dietary vitamin E has a direct effect on the composition of biological membranes.

In addition to their role as cellular and intracellular boundaries, biological membranes also provide a support for enzymes and a number of observations have been made which suggest that the functional integrity of some membrane-bound or membrane-associated enzymes may be impaired in vitamin E deficiency; thus dietary vitamin E affects the activity of the Na-K adenosine triphosphatase of rat liver cell plasma membrane (Machado et al, 1971; Kawai et al, 1974). More recently the activity of some enzymes in the liver of vitamin E-deficient ducklings has been reported (Hulstaert, 1974) to be increased; these were: 5^{1}-nucleotidase, glucose-6-phosphatase, isocitrate dehydrogenase, NADH-tetrazolium reductase, and lactate dehydrogenase. All these enzymes are believed to be membrane-associated.

Although increased activity of some enzymes has been observed in vitamin E-deficiency, while others show a decrease, this is not inconsistent with the view that changes in membrane stability would lead to alterations in the conformation of the membrane, thus further exposing the active site of some enzymes while closing up that of others.

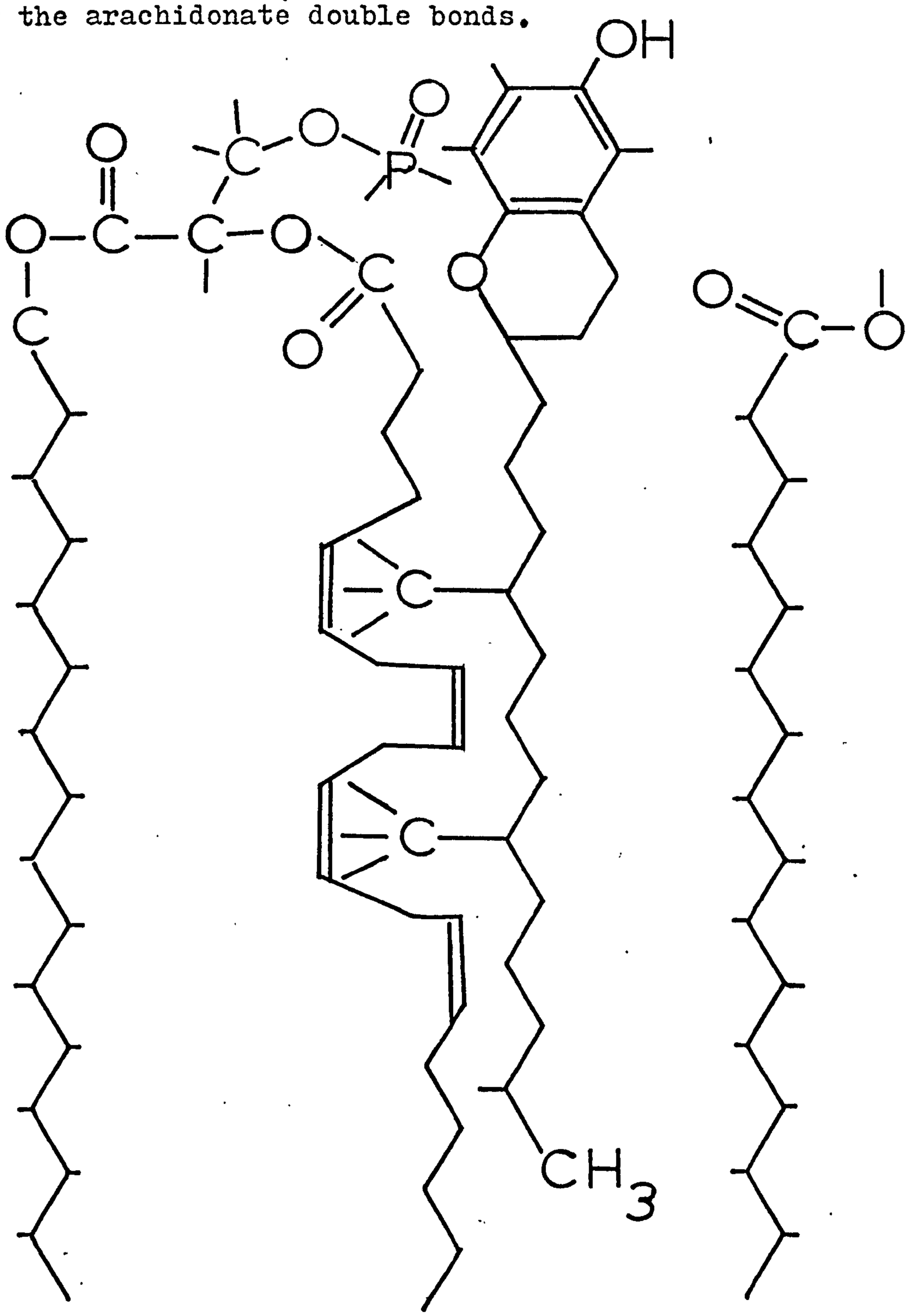
From the results of model-building studies,

Diplock and Lucy (1973) and Lucy (1972) proposed that the observed stabilization of membranes by vitamin E is due to specific physicochemical interactions between the phytyl side chain of α-tocopherol and the fatty acyl chains of polyunsaturated fatty acids, particularly those derived from arachidonic acid (Fig. 6-2). A similar model was proposed by Rosenberg (1967a, 1967b) for interactions between the phytyl chains of chlorophyll and the fatty acyl chains of galactosyl diglycerides in the chloroplasts of Euglena; he observed that all four cis double bonds of arachidonic acid force the chain to assume a curved configuration when packed in a quasiplanar arrangement and cholesterol would then fit (Vandenheuvel, 1963) between the choline of the phospholipid and the end of the curved unsaturated chain.

In the model of Diplock and Lucy (1973) two comparable interactions were proposed between the methyl groups of the phytyl side chain of α-tocopherol and the cis-double bonds of the fatty acid. The methyl group, at C_4, of α-tocopherol can fit into the pocket provided by the first and second double bonds of arachidonic acid (Fig. 6-2). The methyl group, at C_8, of α-tocopherol is then perfectly placed to interact similarly with the third and fourth cis-double bonds of arachidonic acid. In the "complex" formed, the hydroxyl group of α-tocopherol and the polar groups of the phospholipid are coplanar and can participate

FIG. 6-2.

Possible interactions between the arachidonate residue of a phospholipid and the phytyl chain of α-tocopherol in a membrane. The base and phosphate group of the phospholipid interact directly with the polar head of tocopherol, when the methyl groups of tocopherol fit into the 'pockets'created by the arachidonate double bonds.



in polar interactions at the surface of a lipid-bilayer membrane. Furthermore, a close association between the remaining methylene groups of the phytyl and fatty acyl chains is achieved, thus further promoting the stability of the complex through the interaction of London/van der Waals dispersion-attraction forces.

Diplock and Lucy (1973) further suggested that some of the arachidonyl residues of membrane phospholipids might be associated with cholesterol. Therefore, the hypothesis does not require a 1:1 ratio of PUFA to tocopherol, and indeed, the ratio of tocopherol to arachidonate in biological membranes can be calculated to be very small. However, membranes containing large amounts of arachidonic acid would be expected to contain high levels of tocopherol for structural stability, and a deficiency in vitamin E would, according to the theory of Diplock and Lucy, be expected to have certain consequences:

1. The packing of hydrocarbon regions of membranes would be disordered, which would facilitate in a limited manner, the oxidative destruction by free-radical chain reactions, of membrane PUFA <u>in vivo</u>.
2. An increase in membrane permeability to cations and solutes such as that described in the mitochondrial membranes of the liver of rats deprived of vitamin E and selenium (Yeh and Johnson, 1972; 1973) and to a failure of normal compartmentalization of cells.

3. An increase in the degradation of membrane phospholipids *in vivo* by endogenous phospholipases; consequences similar to those of lipid peroxidation would be expected. Phospholipases have been found in mitochondria (Waite *et al*, 1969), in lysosomes (Rahman *et al*, 1970), in the endoplasmic reticulum (Bjornstad, 1966) and in the plasma membrane (Newkirk and Waite, 1971).

4. Alterations in the activity of membrane-associated enzymes as their active sites become either more or less accessible to their substrates because of changes in the membrane architecture.

Recent work by Diplock and Giasuddin has been directed to testing the hypothesis of Diplock and Lucy (1973) using three systems:

a) the effect of α-tocopherol on the growth, permeability to 2-deoxyglucose of, and the uptake of ^{14}C-linoleic acid and ^{3}H-cholesterol by, the plasma membrane of mouse fibroblasts in culture;

b) the permeability to glucose and chromate ions of phospholipid vesicles (liposomes) prepared from phospholipids of varying extent of unsaturation in the presence or absence of α-tocopherol. (Diplock *et al*, 1977);

c) the penetration of tocopherols of varying side-chain length into monolayers at the air-water

interface of synthetic phospholipids of varying unsaturation and length of fatty acid side chain (Maggio et al, 1977).

Preliminary results obtained (Giasuddin, 1976; Giasuddin et al, 1975) indicate a structural relationship in membranes between α-tocopherol, cholesterol and polyunsaturated fatty acids.

CHAPTER 7

INTERACTION OF SELENIUM WITH OTHER TRACE ELEMENTS

Biological interactions occur between selenium and a number of other elements, that render selenium much less toxic than when it is present alone; in addition, the presence of selenium is known to reduce the toxicity of some of these other elements, such as mercury and cadmium.

A. ARSENIC:-

The discovery of the interrelationship between selenium and other elements dates back to the work of Moxon (1938) who reported that the chronic and acute selenoses produced by feeding seleniferous grains containing 15ppm Se could be alleviated, or actually prevented, by the administration of arsenic (5ppm As as sodium arsenite in the animal's drinking water). The symptoms of toxicity in the chosen experimental animal, the rat, were slow growth, mortality, and necrotic and atrophic liver damage. Similar effects were demonstrated by Moxon *et al* (1944) in steers grazed on seleniferous ranges and subsequent work (Moxon *et al*, 1945) showed that arsenate was as effective as arsenite in moderating selenium toxicity. Arsenic was shown (Du Bois *et al*, 1940) to prevent the chronic toxicity of selenium presented as either seleniferous grains, selenite or selenocysteine.

The interaction of arsenic with selenium has also been found to occur with pigs (Moxon, 1941), dogs (Rhian and Moxon, 1943),cows (Moxon et al, 1944) and chicks (Moxon, 1941); in addition, Moxon and Wilson (1944) reported that the inclusion of 10ppm selenium as seleniferous wheat in a hen diet lowered hatchability and that this reduction could be partially prevented by the administration of arsenic in drinking water. At a level of 2.5ppm arsenic, the effect of selenium was still evident and there was a 65% decrease in hatchability; at 5ppm arsenic however, the effect of selenium was almost completely overcome and hatchability was returned to near its normal level.

The mechanism by which arsenic protects against selenium toxicity is unknown. Moxon et al (1945) and Klug et al (1950) have reported that, at chronic toxicity levels, the absorption and retention of selenium do not seem to be influenced by protective amounts of arsenite; however, in rats injected with single subacute doses of selenium, arsenite was shown (Palmer and Bonhorst, 1957) to increase the concentration of selenium in the blood and to decrease it in liver. On the other hand, Klug et al (1950) found that, when rats were fed arsenic (5ppm) in the drinking water and seleniferous corn (10ppm Se) for twelve weeks, the selenium content of the liver and kidneys was substantially increased over that in rats fed the selenium alone. Examination of the food intake revealed that the animals grew more

rapidly and ate more food, thus taking in more selenium when arsenic was included in the diet. When the content of the organs was compared to the intake of selenium, neither liver nor kidney were significantly different and the authors concluded that arsenic counteracts selenium toxicity in some manner other than retarding tissue deposition.

In studies on the elimination of selenium as a volatile compound, Kamstra and Bonhorst (1953) observed that the amount of selenium exhaled, as volatile compounds following the injection of selenite, was greatly decreased in the presence of arsenite. This latter effect of arsenite, however, would be expected to increase, rather than decrease, the symptoms of selenium toxicity unless compensatory mechanisms were operative. Ganther and Bauman (1962) reinvestigated the relationship at subacute levels of arsenic and selenium and showed that arsenite markedly increased the excretion of selenium into the gastrointestinal tract; rats were given subcutaneous injections of sodium arsenite (2.5mg As/kg) followed, ten minutes later, by sodium ^{75}Se-selenite (2.5μCi ^{75}Se). Levels of selenium in the blood, liver and carcass were decreased by arsenite, as were the amounts in the expired air. Kidney levels were increased while urinary excretion of selenium was not affected by arsenite. Levander and Baumann (1966a) showed that the administration of graded doses of arsenic (as $NaAsO_2$) varying from nil to 5mg As/kg body weight in

rats showed a progressive fall in the liver level of selenium and a progressive rise in the amount of selenium recovered from the gastrointestinal tract; the dosage of selenium (as $Na_2{}^{75}SeO_3$) was 2mg Se/kg. This important observation suggested that the bile might be the route of excretion. Subsequent experiments by Levander and Baumann (1966b) on rats with acute billiary fistulas, showed that in a 3-hour collection period, arsenic caused a ten-fold increase in the billiary excretion of selenium. At an arsenic : selenium dosage ratio of 2, arsenic was shown to have an effect on billiary excretion of selenium even at very low levels of dietary selenium (0.02mg Se/kg). This suggests that the billiary route of excretion may have physiological significance when animals are given the minimum selenium levels for dietary requirement and probably explains the finding by Shapiro (1973) of a faecal route of excretion following subcutaneous dosage.

On the basis of these and other experiments, Levander (1972) suggested that "arsenic combined with selenium in the liver, perhaps, in analogy with sulphur chemistry, by reacting with selenol (-SeH) compounds, to form a detoxication conjugate that is readily passed into the bile". The increased billiary excretion of selenium induced by arsenic is counterbalanced by a decrease in the formation of the volatile excretory product dimethylselenide (Ganther and Baumann, 1962).

Arsenic and selenium, in the anionic form are

very similar in structure and chemical reactivities (Hill, 1975). While the basis underlying selenium toxicity is unknown, one of the biochemical lesions that arsenic produces is the uncoupling of oxidative phosphorylation (Wright, 1968) and the uncoupling brought about by arsenate was partially prevented (Wright, 1968) by the presence of selenium in the mitochondrial medium.

The ability of arsenic to prevent the toxic manifestations of selenium in a number of animal species has been discussed. The physiological systems involved in this interaction are as yet unknown; however, the evidence indicates that the retention of selenium in arsenic treated animals may be reduced and the manner of elimination may be changed.

B. CADMIUM

Parizek and Zahor (1956) reported that toxic doses of cadmium caused testicular necrosis in rats and Parizek (1957) later showed that this effect was antagonized by zinc. The first report of selenium involvement in cadmium toxicity was by Kar *et al* (1960) who showed that the cadmium-induced changes in the gonads could be prevented by the administration of either selenium or zinc; Mason *et al* (1964) confirmed these reports and later showed (Mason and Young, 1967) that selenium is approximately 100 times as effective as zinc in preventing testicular injury caused by cadmium.

In addition to the effects of cadmium on the

testis, selenium is capable of preventing all the other known manifestations of cadmium toxicity. Thus, Parizek et al (1968) and Gunn et al (1968) reported decreased mortality of rats given lethal doses of cadmium, when selenium was also administered. Other effects of cadmium which are prevented by selenium include the selective cadmium-induced damage to non-ovulating ovaries (Parizek et al, 1968a), the destruction of the placenta by cadmium salts (Parizek, 1964, Parizek et al, 1968), and an effect of cadmium toxicity which only becomes apparent in the later stages of pregnancy (Parizek, 1965).

Thus, selenium affords general but highly effective protection against cadmium-toxicity; the mechanism by which this is achieved is, at present, obscure. Parizek et al (1969a) reported that the simultaneous administration of selenium with cadmium to rats resulted in elevated blood levels of selenium. Gunn et al (1968) demonstrated the protective effect of selenium against vascular injury to the testes of mice given cadmium, and showed that, in those mice receiving both selenium and cadmium, higher concentrations of both elements were found in the testes than in animals receiving either element alone.

Since the discovery by Rotruck et al (1973) of the function of selenium in the enzyme glutathione peroxidase (see Chapter 9), the relationship between cadmium and selenium has been investigated in experiments

on the effect of cadmium on the enzyme. Omaye and Tappel (1975) injected male rats with 25μmol cadmium chloride/ml/kg; these animals exhibited decreased plasma and testicular glutathione peroxidase activities, testicular atrophy and necrosis, and increased testicular thiobarbituric acid - reactive products. Seven days after the injection, only the plasma enzyme had returned to its normal level. The decrease in testicular GSH-Px activity was not reversed by dialysis against buffer or by the addition of Se as buffered seleno-methionine. The inhibition of testicular GSH-Px *in vitro*, occured at 0.3mM cadmium. All *in vivo* effects were prevented by a prior injection of 25μmol Na_2SeO_3/ml/kg. Omaye and Tappel (1975) suggested that testicular glutathione peroxidase may be the direct or indirect target of cadmium-induced testicular damage, which results in lipid peroxidation.

Gunn *et al* (1968) reported that the administration of selenium significantly diminished the uptake of simultaneously administered ^{109}Cd by the liver and pancreas. It may be that such organ redistribution of cadmium would account, in part, for the protective effects of selenium.

C. MERCURY

The knowledge that selenium moderates the toxicity of cadmium compounds, and the similarity between cadmium and mercuric salts in their effects on

selenium metabolism, led to predictions that the interaction between mercury and selenium would be similar to that between cadmium and selenium. Parizek and Ostadalova (1967) administered a large amount of selenium concomitantly with mercuric chloride and found that the toxicity of mercury was markedly decreased - none of the pathological changes typical of intoxication by inorganic mercuric salts appeared in animals also given selenium compounds. The kidneys and small intestine of female rats given 20 μmol/kg body weight of mercuric chloride were severely damaged 27 hours after administration of the dose and this effect was completely abolished by the administration of the same molar dose of sodium selenite 1 hour after the $HgCl_2$. Further, animals given lethal doses of mercury survived when treated with toxic levels of selenium (Parizek and Ostadalova, 1967). When the excretion of mercury was examined (Parizek et al, 1971), it was found that this was decreased by the administration of selenium, and that in addition to an increased retention of mercury, its distribution in the body was altered. In other experiments, Parizek et al (1969; 1971) showed a decrease in the passage of mercury across the placenta of pregnant female rats into their foetuses, and also a lower level of mercury in their milk; they concluded in a further paper (Parizek et al, 1971a) that the bioavailability of selenium was much lower in mercury-

treated rats. As with cadmium, the level of mercury found in the blood plasma was found to be very substantially increased by the simultaneous administration of selenite or selenomethionine. This effect was observed (Parizek et al, 1971) after the administration of relatively small amounts of selenium and was dependent on the dose given.

Prior to 1972, inorganic salts of mercury were used, usually given by parenteral injection, in most of the investigations of mercury toxicity. If the relationship between selenium and mercury has significance in environmental mercury pollution, then selenium must be shown to have an effect on the toxicity of organic mercury compounds. Potter and Matrone (1974) showed that 40ppm dietary methyl mercury was considerably more toxic to male, weanling rats than 40ppm mercuric chloride and that selenium protected against both forms of mercury toxicity; these results indicate that inorganic mercury salts are bioconverted to organic compounds before utilisation. In addition, Ganther et al (1972) showed that Japanese quail given 20ppm mercury as methylmercury in diets containing 17% tuna survived considerably longer than quail given the same amount of methylmercury in a corn-soya diet. Further, on analysing a number of different batches of tuna for mercury and selenium, Ganther et al (1972) found a striking correlation between the body levels of selenium and mercury; those batches that had little

selenium contained little mercury (1.91ppm Se: 0.32ppm Hg), and when the mercury level was high, the selenium level was elevated also (2.91ppm Se: 2.97ppm Hg). The high mercury levels in the tuna appeared to be less toxic, and it was suggested that the high selenium in tuna was lowering the toxicity to the quail of the added 20ppm methylmercury; the same dose of methylmercury administered in a corn-soya diet proved lethally toxic to the birds. In another experiment using rats, Ganther et al (1972) showed that 0.5ppm Se (as sodium selenite) was completely effective in preventing mortality in animals given 10-25ppm Hg (as methylmercury). These results have since been confirmed by other groups of workers (Stillings et al, 1972; Potter and Matrone, 1973), and a direct metabolic antagonism between mercury and selenium has thus been established; in addition, Koeman et al (1973) reported that high selenium levels were also found to accompany high mercury levels in liver samples obtained from marine mammals and fish-eating marine birds.

The nature of the interaction between selenium and mercury is unclear; it has been shown that selenium compounds can prevent deleterious effects of compounds of mercury, increasing simultaneously their retention in the body and their concentration in certain tissues. Thus, Yamane et al(1977), in an examination of the effect of selenium on the metabolism of mercury

compounds showed that the liver mercury content of rats given mercuric chloride with selenium was significantly greater than in rats receiving $HgCl_2$ alone. Similarly the liver selenium content was greater when mercury was given than for rats given selenium only. The molar ratio of mercury: selenium in the liver was 1 : 1. These results suggested to the authors that selenium interacted directly with $HgCl_2$ to form less toxic products in liver. Sumino et al (1977) confirmed these results and showed that the administration of sodium selenite (69μg) by intravenous injection led to increases in the levels of unbound methylmercury, in the tissues of mice previously injected with CH_3HgCl. Magos and Webb (1977) reported a significant increase in retention of selenium at the injection site when methylmercury was added to selenite solution administered by subcutaneous injection. No similar effect on the retention of mercury was observed, although selenium was shown to cause a decrease in blood content and an increase in brain content of ^{203}Hg; the brain content of selenium was also increased to a lesser extent.

A selenium-induced redistribution of mercury has also been observed in the killifish - Fundulus heteroclitus. Sheline and Schmidt-Nielsen (1975) found that selenium pretreatment caused a redistribution of mercury among the organs of the fish following an i.m. injection of 1ppm MeHgCl in saline; no change in overall

mercury retention was observed. Hg concentration was decreased, significantly in kidney and, to a lesser extent, in liver of Se-treated fish; the level in muscle was considerably higher than that in control fish. Studies using ^{14}C- and ^{203}Hg-labelled CH_3HgCl showed no change in tissue distribution of ^{14}C and ^{203}Hg between Se-treated and control fish, indicating that no breakage in C - Hg bond of CH_3HgCl had occured due to Se-pretreatment.

Experiments by Komiya et al (1977) on the blood of rats simultaneously injected with mercuric chloride and sodium selenite, showed that the reaction product of mercury and selenium in plasma and erythrocytes, was an equimolar compound of the two metals: HgSe. As intermediate products, Se-, Hg- and HgSe- protein complexes were verified by gel-chromatography separation of plasma proteins after experiments both in vivo and and in vitro.

In studies on the toxicity of dimethylselenide, Parizek et al (1971) observed a strongly sex-linked interaction between mercury and dimethylselenide. In male rats, the toxicity of both dimethylselenide and the trimethylselenonium ion was greatly enhanced by the administration of mercuric compounds; this effect was not observed in female rats (Parizek et al, 1974). Thus, when given 1μmol $HgCl_2$/kg body weight, no mortality was induced in control male rats; however, on the simultaneous administration of 1μmol $(CH_3)_2$ Se/kg,

60% of the rats died. Although this dose of dimethyl-selenide was 1/10,000 of the LD_{50} of this substance, as determined by McConnell and Portman (1952), it was high in comparison with levels of methylated selenium metabolites which would be expected in tissues of animals given normal doses of selenium.

D. THALLIUM

The first report of a biochemical interaction between thallium and selenium was by Hollo and Zlatarov in 1960; they reported that death due to thallium poisoning could be prevented by the parenteral administration of selenate. This observation was later confirmed by Rusiecki and Brzezinski (1966) when it was shown that the lethal effects of thallium could also be prevented by oral administration of selenate and that the thallium level in liver, kidney and bones was substantially increased by selenate treatment. The studies of Levander and Argett (1969) revealed that subcutaneous injection of thallium acetate led to an increase in selenium retention in liver and kidney and diminished pulmonary and urinary excretion of selenium. This decreased excretion is similar to that observed after the injection of cadmium (section B of this chapter) or mercuric compounds (section C) and it is probable that the interaction between selenium and thallium may resemble that between selenium and mercury or cadmium.

E. LEAD

The possibility of protection by selenium against lead toxicity was first proposed by Dennis (1971) who indicated that selenium may decrease the toxicity of lead shot in water fowls. Experiments by Rastogi et al (1976) used cutaneous application of lead naphthanate (80-200mg Pb/kg body weight) to produce chronic lead toxicity in rats. Symptoms included reduced growth rate, decreased food consumption and reduced activity of δ-aminolevulinic acid dehydrase, (ALA-D) an enzyme which is inhibited by small amounts of lead (Vallee and Ulmer, 1972). The growth rate and food consumption of rats receiving selenium (5-15ppm) in addition to lead approached normal rate while rats treated with only one of these elements showed hampered growth and low food consumption. The activity of ALA-D in whole blood, liver and kidney and liver cytochrome P-450 enzyme activity were restored to normal levels when selenium was also administered to lead-treated animals.

Levander and Argett (1969) found that lead had no effect on the excretion of volatile selenium in rats. Investigations of the effect of dietary selenium on lead metabolism by Cerklewski and Forbes (1976) revealed that selenium (at levels up to 0.5ppm) was somewhat protective against the toxic effects of 200ppm lead, given as lead acetate. However, at a dietary selenium level of 1ppm, an exaggeration of lead toxicity was observed; tissue lead concentration

was increased in blood, liver, kidney and tibia, and excretion of urinary ALA was increased, but the protective effect of selenium against, lead-induced lowering of ALA-D activity was still observed. Since lead depressed kidney selenium concentration, Cerklewski and Forbes (1976) concluded that "lead may act as an antagonist to selenium metabolism."

The effect of dietary selenium on lead toxicity in the Japanese quail was studied by Stone and Soares (1976); decreased ALA-D activities were observed when 500 and 1000 ppm lead were given to male birds. These decreases were moderated by the administration of 1ppm selenium and, after 85 days, selenium supplementation of lead-containing diets resulted in increased kidney lead levels. Detailed experiments by Levander et al (1977) on the effect of vitamin E status on lead toxicity in the rat, showed that vitamin E deficiency increased the splenomegaly and anaemia in lead-poisoned rats and also increased the mechanical fragility of red cells in these rats; selenium deficiency did not cause any of these effects. Addition of 0.5ppm Se to vitamin E-supplemented diets slightly increased the splenomegaly and anaemia in lead-poisoned rats. In the absence of dietary vitamin E, excess levels of selenium (2.5 and 5ppm) partially prevented these symptoms. Levander et al (1977) concluded that "selenium has little, if any, protective effect against lead poisoning in vitamin E-supplemented

animals. Although excess levels of selenium do have some protective effect against lead poisoning in vitamin E-deficient rats, the levels of selenium that are needed to demonstrate an effect are toxic in themselves."

F. SILVER

In 1951, Shaver and Mason published their findings, showing that silver salts are a stress factor in vitamin E-deficient rats. They described dystrophic lesions, necrotic degeneration of the liver, and high mortality when vitamin E-deficient rats were given either silver nitrate or silver lactate in their drinking water. In a later publication, Mason (1953) stated that the rats' diets contained large amounts of cod-liver oil and indicated that full protection was obtained by administration of vitamin E. Five years later, in similar experiments with chicks, Dam et al (1958) showed that 20ppm silver as silver acetate, had a pro-exudative effect in vitamin E-deficient chicks.

In a detailed study of silver toxicity in vitamin E-deficient rats, Diplock et al (1967) confirmed that silver is a toxic agent in the production of liver necrosis in vitamin E-deficient rats, and that the administration of vitamin E prevented this toxicity. Thus, in rats given a diet nutritionally adequate in vitamin E (120ppm, or 100ppm DPPD) and selenium,

treatment with silver had no effect and the rats were found to have normal livers at the end of the experiment (86 days). However, when α-tocopherol (or DPPD) was withdrawn from the diet, all the rats died between 49 and 64 days; supplementation with selenium (0.05ppm) had little effect whereas the addition of 1.0ppm selenium resulted in 55% protection against the toxic effects of silver. These results indicated (Diplock, 1976) that silver toxicity was due to a metabolic antagonism with selenium. Methionine, at 0.15% in the diet, was found (Diplock et al, 1967) to be partially protective against the necrosis of selenium deficiency but was ineffective against the toxicity of the high levels of silver used (1500ppm Ag as silver acetate). Alterations in the level of dietary fat, or of its degree of unsaturation, revealed that the stress effect was manifested even in the absence of lipid from the diet. The addition of 2.5% of highly unsaturated methyl esters of cod-liver oil fatty acids led to a very slight exacerbation of the silver-induced disease. Experiments on the metabolism of ^{14}C-α-tocopherol showed that there was an accumulation, or decreased rate of utilization, of α-tocopherol in the livers of the silver-poisoned rats compared to the control animals, although treatment with silver did not affect the concentration of tocopherol in the rest of the carcass. As the metabolic study was made during the critical prenecrotic phase of the disease, it was concluded that

lipid peroxidation, which would have occured if the biological antioxidant theory (Tappel, 1962) is to be accepted, was not the causative factor in silver-induced liver necrosis in vitamin E-deficient rats. In addition, Diplock (1976) suggested that, as the turnover of α-tocopherol in the body generally proceeds at a slow rate (Green et al, 1967) the increased levels of ^{14}C-α-tocopherol found in livers of silver-treated rats might indicate that the requirement for tocopherol in the liver of the rats was increased during the pre-necrotic period.

Diplock et al (1967) suggested that the necrotic lesions produced by silver, on the one hand and selenium deficiency on the other, may be biochemically related; it seemed possible that the effect of silver was to lower the biological availability of selenium so that, in the absence of vitamin E, a condition identical to dietary liver necrosis was being produced. In order to test this hypothesis, a detailed pathological examination by light and electron microscopy of the silver-induced necrosis was undertaken (Grasso et al, 1969) and direct comparison with the necrosis caused by dietary deficiency of selenium and vitamin E was carried out. Addition of silver acetate to either the diet (130-1000ppm Ag) or drinking water (1500ppm Ag) of weanling rats fed a vitamin E-deficient diet resulted in necrotic degeneration of the liver after about 14

days. In the prenecrotic stage (11-13 days), the hepatocellular changes were characterised by nuclear vacuolation, by an increase in the number and size of lysosomes, and by their movement away from the peri-billiary area. The mitochondria were enlarged and they assumed irregular, often bizarre shapes.

The hepatocellular necrosis appeared initially in the centrilobular area and rapidly spread into the entire lobule. Degeneration of nuclei, mitochondria and endoplasmic reticulum appeared to occur simultaneously, and numerous large lysosomes containing electron-dense material, thought to be silver, were observed following macrophage infiltration. Rats given dietary vitamin E as well as silver acetate had completely normal livers.

The mitochondrial changes observed in livers of silver-poisoned rats had some similarities to those seen in rats fed a Torula-yeast diet deficient in vitamin E and selenium (Fite, 1954) and these observations were taken to provide confirmatory evidence that silver toxicity causes a selenium deficiency in the absence of vitamin E.

The pro-exudative effect of silver in vitamin E-deficient chicks was first described in 1958 by Dam et al. Further investigation by Bunyan et al (1968) confirmed that 0.15% of silver acetate in the drinking water produced silver toxicity in chicks given a fat-free casein-gelatin diet, deficient in vitamin E. After two weeks, the chicks were found to have colourless

exudates, mainly in the pectoral region and partly in the peritoneal and pericardial spaces. The administration of vitamin E and selenium, separately or together, failed to prevent this condition, but in the presence of 0.5% of added methionine, vitamin E was completely protective. When lard was added to the diet and the chicks were also given silver, green exudates were observed in addition to the colourless ones. The green coloration, but not the colourless exudate, was prevented by vitamin E and selenium, separately or together. Methionine enhanced the green exudative condition but, when combined with vitamin E, prevented both types of exudate. The basal diet was found to be deficient in sulphur aminoacids since, when given alone, it produced a high incidence of dystrophy of the breast muscle.

It appears, therefore, that silver is capable of antagonizing selenium in vitamin E-deficient chicks and that an adequate or high level of sulphur amino acids is necessary to prevent the exudative condition in the presence of dietary vitamin E. Diplock (1976) suggested that the maintenance of adequate levels of tissue glutathione peroxidase activity necessitated the provision of substantial amounts of dietary sulphur amino acids for the maintenance of tissue levels of glutathione.

In experiments in turkey poults, Peterson and Jensen (1972) showed that the addition of 900ppm Ag as

silver acetate to a corn-soy, fish-meal diet produced microcytic, hypochronic anaemia, a circle-shaped appearance of the heart, and gizzard muscular dystrophy; these lesions were prevented by the addition of dietary selenium.

Silver toxicity in cattle has been suggested by Partschefeld (1974) in a report of muscular dystrophy of the type usually associated with selenium deficiency, which was observed in calves in regions with a large amount of silver mining activity. Investigation revealed that milk given to the calves contained adequate levels of selenium but a low vitamin E content. However, the silver content of the milk was believed to be high, although no precise data were given.

PART II

INTRODUCTION TO EXPERIMENTAL WORK AND DEVELOPMENT OF METHODS

CHAPTER 8

REDUCED METABOLITES OF SELENIUM

It is well-established that animals given large doses of inorganic selenium salts such as sodium selenite are able to convert these compounds into suitable excretory products (Schultz and Lewis, 1940; McConnell, 1942). This detoxication is achieved by the formation of methylated products which are either exhaled (McConnell and Portman, 1952) or are excreted via the urine (Byard, 1969; Palmer et al, 1969). McConnell showed that within 24 hours, 3-10% of an administered dose of sodium ^{75}Se-selenate appeared in the exhaled air of rats to which it was given by intravenous injection. Schultz and Lewis found that within 8 hours, 17-52% of a dose of sodium selenite given by subcutaneous injection, was excreted via the lungs; they trapped the expired compound in sulphuric acid and determined the selenium content colorimetrically by comparison with known standards using the codeine-selenite reaction (see Chapter 10). The conclusive work of McConnell and Portman who demonstrated that dimethyl selenide was exhaled without further metabolic change, shortly after it had been administered, lent further support to the case that inorganic selenium salts are metabolically converted to dimethyl selenide for respiratory excretion.

Early work which led to the recognition of selenium

as an essential trace element (Schwarz, 1944; Schwarz and Foltz, 1957; Dam et al, 1957) involved the important discovery that selenium is an integral part of a component in baker's yeast, called factor 3, which is capable of preventing liver necrosis in rats and exudative diathesis in chicks (Scott et al, 1957). Both of these conditions are now recognised as symptoms of selenium deficiency. Although factor 3 is incompletely characterized, it has been shown (Schwarz and Foltz, 1957) to contain organoselenium compounds which are more active per mole of selenium than inorganic selenium salts, in protecting against the deficiency symptoms. However, the fact that salts such as sodium selenite and sodium selenate can be and are used to prevent or reverse deficiency signs indicates that there must exist a pathway for the bio-conversion of inorganic salts to biologically active selenium compounds.

Since we know that toxic doses of sodium selenite or sodium selenate are eventually excreted as methylated organic derivates, a study of this pathway might give an indication of how inorganic selenium salts are converted to biologically active selenium compounds. [The synthesis of dimethyl selenide from sodium selenite or selenious acid is chosen for discussion here since the reaction can be carried out under laboratory conditions and the end-product is readily isolated.]

In a series of independent experiments, other workers (Diplock et al, 1968) found a form of acid-volatile selenium in liver cell fractions. Further work (Caygill et al 1971) suggested that the acid-volatile selenium was particularly associated in adequately-fed rats, with the mitochondria and the smooth endoplasmic reticulum. Four dietary treatments were investigated using Caesarian-derived male Wistar rats, and the liver intracellular distribution of ^{75}Se, $^{75}Se^{2-}$, and $^{75}SeO_3^{2-}$ formed from orally administered $Na_2{}^{75}SeO_3$ was studied (Diplock, et al, 1971). Subcellular fractionation, using zonal centrifugation techniques and checked by marker enzymes, was carried out in order to separate the lysosomal, mitochondrial, smooth- and rough-surfaced endoplasmic reticulum and soluble fractions. The dietary treatments studied were:

a) Vitamin E-deficient diet for 3 months; re-fed with vitamin E during the terminal 5 days.

b) Vitamin E-deficient diet.

c) Adequate diet.

d) Vitamin E- and selenium-deficient diet; re-fed with vitamin E during the terminal 5 days.

In the rats given the adequate diet (c), selenide was found to be particularly associated with liver mitochondria and, to a lesser extent, with smooth endoplasmic reticulum. Little selenide was found in vitamin E-depleted rats and no association with mitochondria was established; however, on re-feeding

vitamin E to the animals (groups (a) and (d)), the normal pattern was restored.

These results supported the hypothesis (Diplock and Lucy, 1973) that the active form of selenium could be selenide located in non-haem iron-containing proteins and that the acid-volatile selenium detected in liver-cell fractions was H_2Se from tissue selenide. However, the possibility could not be excluded that alkyl selenide located in subcellular organelles might release H_2Se on acidification and subsequent rupture of these organelles. Experiments were therefore undertaken (Diplock et al, 1973) to attempt to distinguish between minute quantities of $H_2{}^{75}Se$ and $(CH_3)_2{}^{75}Se$. These workers found that H_2Se in the trace amounts utilised was extremely susceptible to oxidation, usually to the red allotropic form of elemental selenium. Preliminary experiments were performed using $H_2{}^{75}Se$ generated by bubbling nascent hydrogen through a solution of $Na_2{}^{75}SeO_3$.

$$Na_2{}^{75}SeO_3 \xrightarrow{Zn + HCl} Na_2{}^{75}Se \xrightarrow{HCl} H_2{}^{75}Se$$

Quantitative trapping of the hydrogen selenide in 0.1M silver nitrate solution was achieved using polythene and polystyrene apparatus with O_2-free nitrogen as carrier gas.

$$H_2{}^{75}Se + 2AgNO_3 \longrightarrow Ag_2{}^{75}Se + 2HNO_3$$

In model experiments, $(CH_3)_2{}^{75}Se$ was generated using the system described by Ganther (1966) for the bio-

synthesis of $(CH_3)_2Se$ <u>in vitro</u> in a cell-free system derived from mouse liver. 8M HNO_3 was found to be an excellent trapping medium for dimethyl selenide, but comparatively ineffective for hydrogen selenide (H_2Se). The means of distinguishing between $H_2{}^{75}Se$ and $(CH_3)_2{}^{75}Se$ established by these experiments was then employed to identify the acid-volatile ^{75}Se derived from liver homogenates and subcellular organelles. Carrier gas was bubbled through liver homogenates before and after acidification with concentrated HCl. The volatile selenium was trapped, in a sequence of experiments, in 0.1M silver nitrate solution, 8M nitric acid solution, and water. The results obtained are shown in Table 8-1 and they establish that the acid-volatile protein-bound selenium of rat liver is selenide which, on acidification, liberates hydrogen selenide (H_2Se).

The knowledge that 18-20% of the selenium present in normal tissues exists as selenide, and that inorganic selenium compounds can be converted to organic metabolites, led to further investigations into the pathway for the biosynthesis of organo-selenium compounds.

A convenient pathway for study is the synthesis of dimethyl selenide from sodium selenite, a major metabolic route for detoxifying subacute doses of selenite (Schultz and Lewis, 1940; McConnell, 1942). This volatile selenide is readily isolated and its formation involves a six-electron reduction in the oxidation state of selenium, making this system a useful model for other reductive pathways of selenium utilization.

TABLE 8-I Diplock et al, 1973

Identification of acid-volatile protein-bound selenium from rat liver homogenates

In Trials 1 and 2, triplicate pairs, and in Trial 3, quadruplicate pairs of adequately fed rats were given three consecutive daily intraperitoneal doses of $Na_2{}^{75}SeO_3$ (50μCi ≡ 9μg Se) in 0.1mL of 0.9% (w/v) NaCl and killed on the fourth day. Then 10% (w/v) homogenates were prepared in 0.25M sucrose solution containing 100μg of α-tocopherol/ml and 5mM-mercaptoethanol. Samples (0.5mL) of the homogenates were placed in the reaction vial, O_2-free N_2 was passed for 10 minutes and 1mL of HCl added. Carrier gas was passed for a further 10 min and trapping of the acid-volatile products was investigated in the agents shown. Values given are mean values ±SD, with numbers of observations given in parentheses.

Trapping agents	0.1M$AgNO_3$	8MHNO_3	Water	0.1M$AgNO_3$	8MHNO_3	0.1M$AgNO_3$	8MHNO_3
Initial ^{75}Se in homogenate (c.p.s)	627±13(6)	663±7(6)	650±4(6)	1017±13(6)	1077±21(6)	1529±15(8)	1401±47(8)
^{75}Se volatilised on acid treatment (^{75}Se) (% of initial ^{75}Se)	16 ± 0	17 ± 1	18 ± 0	19 ± 2	20 ± 2	14 ± 0	13 ± 1
^{75}Se trapped in agent on acid treatment (% of ^{75}Se volatilized)	91 ± 6	8 ± 2	9 ± 3	91 ± 2	11 ± 3	91 ± 2	3 ± 0

A. Pathway for Selenium Reduction:

Present knowledge of the pathway in liver for the metabolism of selenium is due almost exclusively to the work of Dr. Howard Ganther at the University of Wisconsin at Madison. The process whereby reduction of selenium *in vivo* may be achieved is inferred from studies carried out *in vitro* with cell-free extracts of mouse liver. In his first published report on this subject, Ganther (1966) confirmed several important characteristics of the system.

1. The process is enzyme catalyzed and activity is proportional to the protein concentration of the liver cell extract.

2. Anaerobic conditions are essential and incubation under N_2 effected a tenfold increase in activity compared to incubation under air.

3. There is an absolute requirement for glutathione which cannot be replaced by dithiothreitol, mercaptoethanol, thioglycoate or L-cysteine.

4. The system is NADPH dependent even when the level of reduced glutathione is kept high.

5. The methyl donor is S-adenosyl methionine, and methionine itself is relatively ineffective in the system *in vitro*.

6. The system is strongly inhibited by arsenite (50% inhibition at 10^{-6}M) in the presence of a large excess of monothiols and is also inhibited by cadmium.

7. Coenzyme A, adenosine triphosphate and Mg^{2+} are also required for maximum activity.

B. Pathway for the synthesis of dinethyl selenide

The conditions used by Ganther, under which the conversion of sodium selenite *in vitro* to dimethyl-selenide may be achieved, were important in providing a background for further investigations into the reaction mechanism of this conversion. The necessity for anaerobic conditions indicated that oxidant-labile selenium compounds are probably produced as inter-mediates in the pathway. The absolute requirement for glutathione suggested that its function is not merely as a reducing agent, but that derivatives of it are involved as metabolites in the pathway. The requirement for NADPH suggests that a NADPH-dependent reductase is one of the enzymes involved; and the need for S-adenosyl-L-methionine indicates the involvement of a methyltransferase.

The reaction of selenious acids with thiols was next investigated. Painter, in a review in 1941, proposed that the reaction could be described by the following equation:

$$4RSH + H_2SeO_3 \longrightarrow RSSeSR + RSSR + 3H_2O \qquad (1)$$

Ganther (1968) confirmed that the combining ratio for several thiols and selenious acid was 4 : 1, by measuring the moderately stable selenotrisulphides (RSSeSR) which have an enhanced absorption between

260-380nm. The identity of the products was established by thin layer chromatography and column chromatography, using chelated copper as the stationary phase, which permitted isolation of the selenotrisulphide.

Sandholm and Sipponen (1973) investigated the stability of the selenotrisulphide (GSSeSG) that is formed from glutathione and selenite. They showed that the compound was unstable and decomposed to elemental selenium and oxidised glutathione, and that the decomposition was greater at high pH. The ratio of GSH : selenite greatly influenced the products that were formed. When the ratio was low (< 4) the main products were GSSG, Se and GSSeSG. With a relatively higher amount of GSH, the main products were Se and GSSG, indicating that the trisulphide decomposed very rapidly. Ganther (1971) found that GSSG and elemental Se could also be liberated from GSSeSG by the addition of very small amounts of highly purified yeast glutathione reductase at pH7.

When physiological conditions of pH and reactant concentrations were simulated and 10^{-6}M ^{75}Se-labelled selenite was treated with GSH (4×10^{-3}M) at pH7, followed by 50mM iodoacetate (Ganther, 1971), the major selenium compound formed was the Se-carboxymethyl derivative of glutathione persulphide (GSSeH).

Ganther suggested that the persulphide was formed by reduction of the initial selenotrisulphide product by excess GSH.

Glutathione reductase was shown (Ganther, 1971) to be inactive when NADPH was excluded from the reaction medium or when selenodicysteine was substituted for selenodiglutathione. The reduction of GSSeSG was therefore catalysed by a NADPH-dependent glutathione reductase, forming GSSeH as the major selenium product.

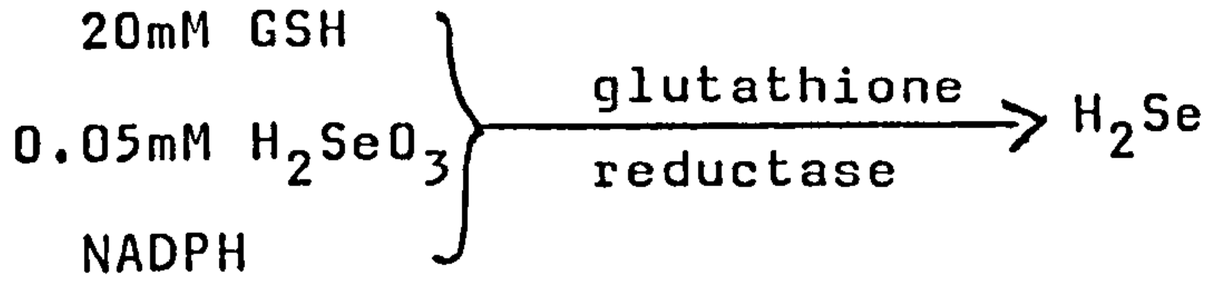
$$GSSeSG + NADPH + H^{+} \xrightarrow[\text{reductase}]{GSH} GSH + GSSeH + NADP$$

The selenopersulphide is unstable and rapidly decomposes to GSH and elemental selenium. In the presence of 50mM iodoacetate however, this compound is converted to the carboxymethyl derivative which was identified (Ganther, 1971) by thin layer electrophoresis, thin layer chromatography and gel filtration.

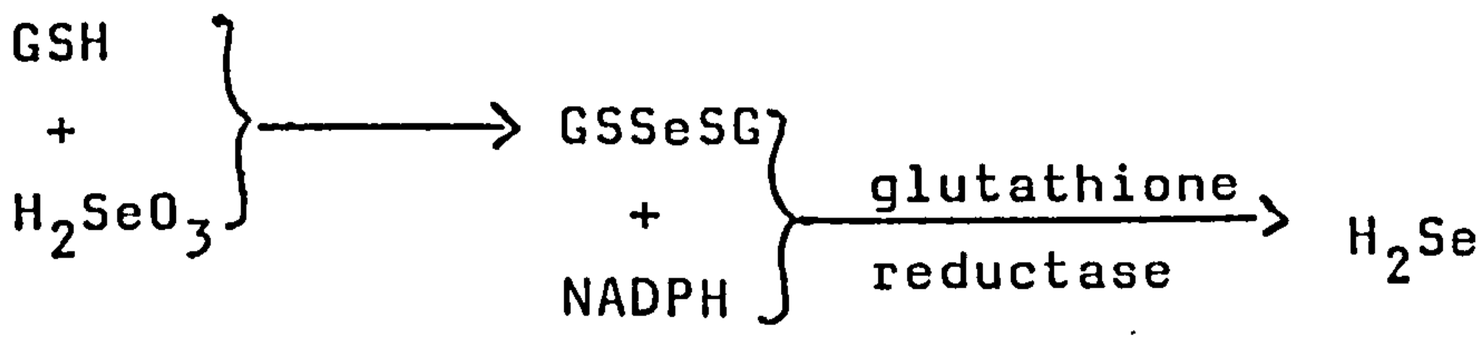
$$GSSeH \xrightarrow{I\text{-}CH_2COOH} GSSe - CH_2COOH + HI$$

Further work (Hsieh and Ganther, 1975) showed that acid-volatile selenide was produced when 20mM GSH was incubated in an anaerobic system with 0.05mM sodium selenite, a NADPH generating system and microgram quantities of highly purified yeast glutathione reductase. The product was analysed using the technique described by Diplock et al (1973) who described the use of silver nitrate solution as a quantitative trapping agent for hydrogen selenide and nitric acid solution for dimethyl selenide. The acid-volatile selenide obtained from the

incubation medium of Hsieh and Ganther (1975) was trapped in $AgNO_3$ solution and thus shown to be hydrogen selenide. Thus:-



Selenodiglutathione (GSSeSG) was also readily converted to H_2Se by glutathione reductase and NADPH in the absence of GSH:



Black (1963) isolated glutathione reductases from a variety of sources and, in view of the similarity of all these enzymes, it appears likely that the enzyme-catalyzed reactions with selenium which have been demonstrated *in vitro* using purified yeast enzyme also occur *in vivo* catalyzed by the enzyme in animal tissues. Ganther and Hsieh (1973) therefore proposed a scheme for the biosynthesis of selenides from selenite (Table 8-2) which is essentially that proposed by Diplock *et al* (1973).

TABLE 8-2

PATHWAY FOR THE BIOSYNTHESIS OF SELENIDES FROM SELENITE

$$H_2SeO_3 + 4GSH \longrightarrow GSSeSG + GSSG + 3H_2O$$

(Se oxidation state +4) (Se oxidation state +2)

$$GSSeSG + NADPH + H^+ \xrightarrow[\text{reductase}]{\text{glutathione}} GSSeH + GSH + NADP$$

(Se oxidation state +2) (Se oxidation state 0)

$$GSSeH + NADPH + H^+ \xrightarrow[\text{other reductase}]{\text{glutathione or}} H_2Se + GSH + NADP$$

(Se oxidation state 0) (Se oxidation state -2)

H_2Se

- → Acid-labile protein Se^{2-}
- → S-adenosyl methionine + methyl transferase
  - → (1) $(CH_3)_3\ Se^+$
  - → (2) $(CH_3)_2\ Se$

The further reduction of GSSeH to H_2Se was suggested because in earlier experiments, Ganther (1971) made careful measurements of NADPH oxidation in the absence of iodoacetate and found that more than one mole of NADPH was oxidized per mole of GSSeSG. This suggested that further reduction of GSSeSG to H_2Se could occur to some

extent, either by the direct action of glutathione reductase on GSSeSG or indirectly by reaction with GSSeH, which can be considered as an analogue of GSSG with (HSe-) replacing one of the glutathione moieties (GS-).

C. Final Reduction and methylation

Ganther and Hsieh (1973) reported their investigations on the final reduction and methylation steps and their attempt to separate the enzymes involved in the two processes. Both kidney and liver were examined and it was found that in kidney, the biosynthesis of dimethylselenide from selenite occurs entirely in the soluble fraction; liver however was found to require the microsomal fraction for optimal activity.

The kidney soluble system was separated by Sephadex G-75 chromatography into four fractions:

Fraction A : proteins larger than haemoglobin

Fraction B : haemoglobin

Fraction C : proteins of around 30,000 molecular weight

Fraction D : proteins of around 10,000 molecular weight.

These fractions were tested alone or in various combinations and only Fraction C was shown to have activity by itself. Addition of either Fraction A or B increased the activity of C while D was ineffective.

Fraction C, like the total soluble fraction, was susceptible to inhibition by arsenite but higher levels were needed to achieve complete inhibition, than were required to inhibit the total fraction. In addition, the activity of Fraction C, preincubated with arsenite, was completely restored following dialysis, whereas that of the soluble fraction was not. This suggests that, in the case of Fraction C, arsenite might inhibit the system by reacting with selenide intermediates in a stoichiometric fashion, whereas the irreversible inhibition of the whole fraction and inhibition at very low levels of arsenite are consistent with the binding of arsenite to a protein acting catalytically at a stage before the transmethylase in the synthesis of dimethylselenide. It thus appeared that the activity of Fraction C was due to an arsenite-insensitive methyl transferase acting on small amounts of hydrogen selenide which can be produced non-enzymically by an excess of glutathione (i.e. in the absence of Fractions A and B), or in larger amounts by a pathway involving a reductase, presumably contained in Fractions A and B.

On further purification of Fraction C by DEAE cellulose chromatography, a Fraction C-IIIa was obtained by Ganther and Hsieh (1973) which appeared to be the methyl transferase and accounted for 95% of the total activity of Fraction C. Addition to it of yeast glutathione reductase effected a stimulation of

dimethylselenide production from selenite, which was thought to be due to the increased production of H_2Se.

When liver was examined (Ganther and Hsieh, 1973) the methyltransferase was found to be located in the microsomal fraction and microsomes were active by themselves when high levels of glutathione ($2 \times 10^{-2}M$) were supplied. Furthermore, they showed no dependence on NADPH, indicating that they rely entirely on selenide produced non-enzymatically. Addition of the liver soluble system to microsomes stimulated dimethyl selenide synthesis and, upon further purification of the liver soluble system by Sephadex G-75 chromatography, this stimulatory agent was found to be located in a Fraction A of the soluble system. The effect of Fraction A was largely NADPH-dependent (Hsieh and Ganther, 1977) and was synergistic at physiological GSH levels ($2 \times 10^{-3}M$) whereas at higher levels, it was merely additive.

Experiments to determine whether yeast glutathione reductase could stimulate the effect of Fraction A on liver microsomes yielded variable results, which were again dependent on the GSH levels used. At low (i.e. 0.6 - $2 \times 10^{-3}M$) glutathione levels, the degree of stimulation achieved decreased with increasing GSH concentration. At high GSH levels (i.e. > 2mM) no stimulatory effect was obtained with glutathione reductase.

Hsieh and Ganther (1977) after separating the liver soluble fraction by Sephadex G-75 chromatography, tested the fractions singly and in combinations for their ability to synthesize dimethyl selenide: only liver fraction C had ability by itself, and the addition of Fraction A stimulated this activity while little or no effect was observed with Fractions B and D. When Fraction A was tested using ^{75}Se-sodium selenite, loss of radioactivity from the reaction flask suggested that this fraction contained glutathione reductase which catalysed the production of hydrogen selenide. This suggestion agreed with the observation that Fraction A stimulated the synthesis of dimethyl selenide by liver microsomes.

Purification of liver Fraction A by DEAE cellulose chromatography yielded four fractions, two of which were active. A_{II} contained the glutathione reductase activity in Fraction A, and A_{III} contained a non-specific NADPH-dependent disulphide reductase, as described by Tietze (1970). In view of the complexity of these fractionation studies by Ganther et al, the distribution of activity in liver and kidney fractions which they observed is summarised in Tables 8-3 and 8-4.

D. Present Experiments on Selenium Metabolism

The system of Ganther (1966) was used in the experiments to be described here, with some modifications

TABLE 8-3

ACTIVITY OF LIVER FRACTIONS IN THE SYNTHESIS OF DIMETHYLSELENIDE

	SEPHADEX G-75 chromatography	ABILITY TO SYNTHESIZE DIMETHYLSELENIDE?	DEAE-CELLULOSE CHROMATOGRAPHY	CONTENTS
LIVER	A : Proteins larger than Hb	X	→ A_{II} :	GLUTATHIONE REDUCTASE
			→ A_{III}:	NADPH-DEPENDENT DISULPHIDE REDUCTASE
	B : Hb	X		
LIVER SOLUBLE FRACTION	C: Proteins ca. 30,000 mol.wt.	✓	→ C_{IIIa}:	METHYLTRANSFERASE ACTING ON NON-ENZYMICALLY PRODUCED H_2Se
	D : Proteins ca. 10,000 mol.wt.	X		
	A + C	✓		
MICROSOMES +20mM GSH		✓		Se-methyltransferase acting on enzymically produced H_2Se from cytosol enzymes or non-enzymically produced H_2Se when [GSH] is high.
	MICROSOMES + SOLUBLE FRACTION	✓		
	MICROSOMES + FRACTION A (2mM GSH)	✓✓		

(After Hsieh & Ganther 1977)

TABLE 8 - 4

ACTIVITY OF KIDNEY FRACTIONS IN THE SYNTHESIS OF DIMETHYLSELENIDE

	SEPHADEX G-75 CHROMATOGRAPHY	ABILITY TO SYNTHESIZE DIMETHYLSELENIDE?	DEAE-CELLULOSE CHROMATOGRAPHY	CONTENTS
KIDNEY —100,000 x g→ KIDNEY SOLUBLE FRACTION	A: Proteins larger than Hb	X	→ A_{II}:	Glutathione reductase
			→ A_{III}:	Non-specific disulphide reductase
	B: Hb	X		
	C: Proteins ca. 30,000 mol.wt.	✓	→ C_{IIIa}:	Methyltransferase
	D: Proteins ca. 10,000 mol. wt.	X		
	A + C	✓		
	B + C	✓		

(after Ganther & Hsieh 1973)

to investigate the influence of dietary treatments on pathways of selenium reduction.

Incubations were carried out at 37°C in polystyrene vials with polythene connecting tubes. Several incubations were done simultaneously using a manifold and distributor as shown in Fig. 8-1. Details of the arrangement of each incubation tube are shown in Fig. 8-2 and the vials shown were described by Diplock et al (1973). The optimum lengths of the polythene tubings (1) and (2) were determined in preliminary experiments and found to be 190mm and 90mm respectively. The length of polythene tubing (1) ensured that fluid added through the syringe was thoroughly mixed with the reaction mixture already in the tube; thus, samples of the reaction mixture withdrawn during the incubation should be representative of the total contents of the tube. A length of 90mm was used for polythene tubing (2) in order to avoid contamination of the long hypodermic needle, which was not disposable, by ^{75}Se from the incubation mixture; in addition, the use of this length enabled some variation in the point at which carrier gas was introduced to the reaction vial: thus N_2 could either be blown across the surface of the fluid or bubbled through the reaction mixture.

E. PREPARATION OF EXTRACTS

Male Wistar rats given various dietary treatments were killed by cervical dislocation and their livers removed and placed in ice-cold 0.25M sucrose solution

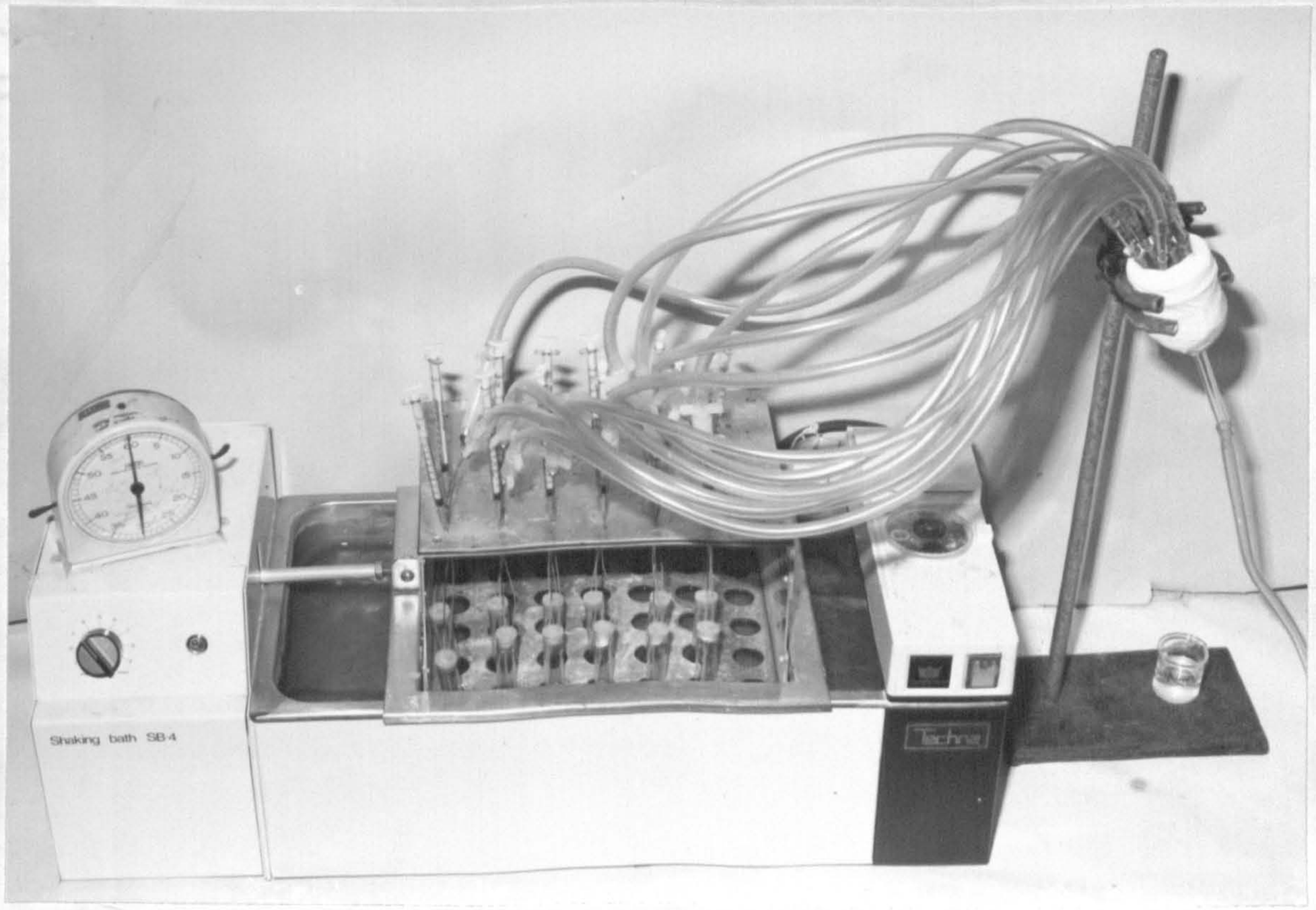


Fig. 8-1. Apparatus for the generation of dimethyl selenide

The reaction mixtures were placed in polystyrene vials in a water bath at 37°C.

Hypodermic syringes connected to polythene tubing, leading into the reaction tubes, enabled additions to or withdrawals from the incubation mixture to be made.

Anaerobic conditions were maintained by flushing O_2-free N_2 through the vials.

INCUBATION TUBE FOR THE SYNTHESIS OF DIMETHYL SELENIDE.

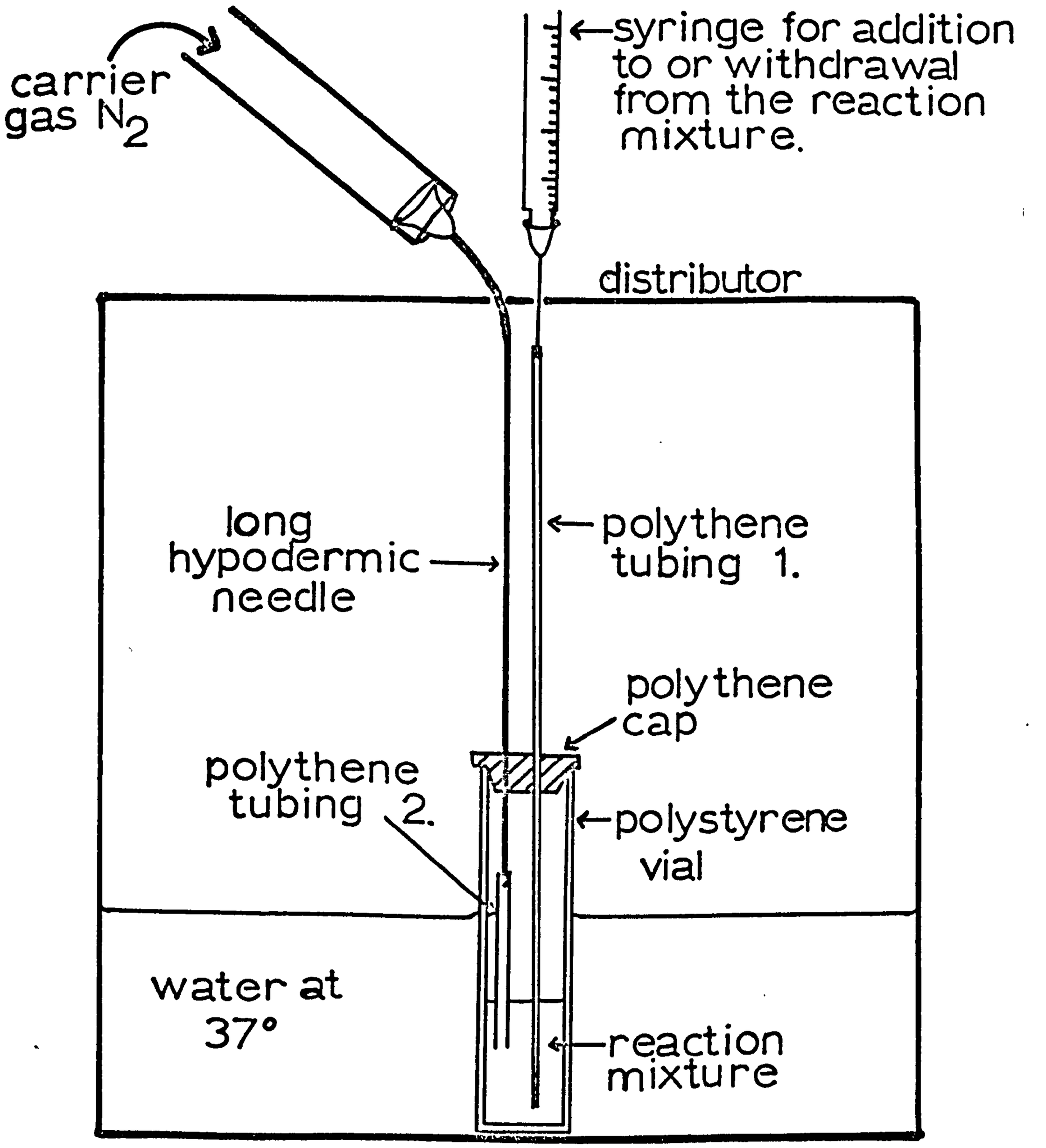


Fig. 8-2.

Arrangement of individual incubation tubes for the synthesis of dimethyl selenide.

containing 10^{-4}M EDTA. After weighing the livers, 25% homogenates were prepared in the same medium using four passes of the homogenizer described by Caygill et al (1971) and the homogenates were centrifuged at 9000g for ten minutes to remove mitochondria and nuclear debris. The volume of the 9000g supernatant was measured and it was then centrifuged at 100,000g for one hour using the MSE Superspeed '65' ultracentrifuge to separate the microsomal and soluble fractions. Microsomes were suspended in the sucrose-EDTA medium and re-centrifuged for another hour at 100,000g; the washed microsomes were resuspended in sucrose-EDTA solution of same volume as the 9000g supernatant from which they were prepared. Aliquots of a) 9000g supernatant, b) 100,000g supernatant, c) washed-microsomal suspension and d) a combination of b) and c) were used in studies of the synthesis of dimethyl selenide. The incubation medium used was based on that of Ganther (1966) and details are given in Table 8-5.

TABLE 8-5

Incubation medium for DMSe generation

	(μmols/3.0mls final volume)
Sodium phosphate buffer	100
Glutathione	60
Magnesium Chloride	40
Adenosine triphosphate	12
S-adenosyl-L-methionine	2
Sodium ^{75}Se-selenite ($Na_2\ ^{75}SeO_3$)	0.15
Coenzyme A	0.6
Ethylenediaminetetraacetic acid	3
$NADP^+$	0.4
Glucose-6-phosphate	6
Glucose-6-phosphate dehydrogenase	6 units

The complete medium without selenite was incubated at 37° under N_2 with aliquots of the liver extract. After five minutes, the radioactive selenium was added to start the reaction. Volatile products were trapped in 8M nitric acid (Diplock et al (1973)) and their radioactivity determined; each sample was counted three times for a period of ten minutes and mean values were used in the calculations. At the end of the incubation period, (ca. 20 mins.) 0.3ml of 5M sodium hydroxide solution was added to the mixture to stop the reaction, and gassing was continued for a further ten minutes. The residual ^{75}Se counts in the reaction tubes were also determined. Between 90 and 95% of the radioactive selenium was recovered in the volatile fractions and residual tube contents. Incomplete recoveries resulted from small losses of volatile products. The amounts of volatile selenium produced were therefore calculated by difference from the values of residual radioactivity in the reaction tubes.

The following experiments were carried out in order to establish the optimum conditions for the generation of dimethyl selenide in vitro.

EXPT. (1)

Nature of the Selenite source and its effect on DMSe synthesis

Ganther (1966) reported that the in vitro synthesis of dimethyl selenide was strongly inhibited by arsenite. This observation further strengthens the biological link

between arsenite and selenium. Arsenite is known to antagonize or protect against both the chronic and acute toxicity of selenium (Moxon 1938; Kamstra & Bonhorst 1953). In addition, arsenite prevents the formation of volatile selenium in animals injected with selenite (Kamstra and Bonhorst 1953; Ganther and Baumann 1962). Since radioactive ^{75}Se decays to arsenic, the effect of diluting sodium ^{75}Se-selenite with carrier Na_2SeO_3 was investigated since it was thought that the arsenic content of the ^{75}Se used might be sufficiently large to inhibit the selenium volatilisation.

Procedure

Two adequately fed rats (250-300g) were killed and their livers removed; 15% homogenates (3gm liver; 17mls medium) were prepared in ice-cold 0.25M sucrose-10^{-4}M EDTA solution. The homogenates were centrifuged at 9000g for ten minutes at 4°C; the supernatant fraction from this centrifugation was used for the experiments.

The incubation medium (see above) without selenite was added to 0.3ml of the 9000g supernatant in the reaction vials, which were then placed in a shaking water bath at 37°. The mixture was gassed for 15 min. with O_2-free N_2. Two aliquots of each supernatant were tested using ^{75}Se-sodium selenite to start the reaction, while a mixture of ^{75}Se-Na_2SeO_3 and carrier selenite (i.e. 0.007μmol. ^{75}Se with 0.18μmol carrier Se per tube) was used in two other experimental vials. The

reaction was stopped after thirty minutes by the addition of 0.5ml of 5N NaOH solution. Gassing was continued for a further ten minutes after which the residual ^{75}Se radioactivity was determined. Loss of ^{75}Se from the reaction tubes was calculated and results are shown in Table 8-6.

TABLE 8-6

Synthesis of dimethylselenide using ^{75}Se-sodium selenite and $Na_2{}^{75}SeO_3 + Na_2SeO_3$ as selenite source

Nature of selenite used	Initial Count* (c.p.m. x 10^{-3})	Residual count* (c.p.m. x 10^{-3})	% of initial count lost
^{75}Se only	10.7	10.4	2.5
^{75}Se only	10.3	9.7	5.4
^{75}Se + carrier Se	2.2	1.1	48.8
^{75}Se + carrier Se	2.1	1.0	51.2
^{75}Se only	10.5	10.2	1.8
^{75}Se only	10.2	9.9	1.9
^{75}Se + carrier Se	2.1	1.3	36.6
^{75}Se + carrier Se	2.2	1.4	32.1

*Each figure represents the mean of four determinations.

Relatively higher percentage losses were obtained with a mixture of ^{75}Se and carrier selenite than when ^{75}Se-selenite only was used. Observations that arsenite inhibits dimethyl selenide synthesis have been reported earlier in this chapter and the knowledge that ^{75}Se undergoes 100% decay to ^{75}As (half-life 118.5 days) by electron capture and gamma emission suggested an explanation for the results in Table 8-6, i.e. the decay product of ^{75}Se was acting as an inhibitor to the reaction under study.

In all subsequent experiments therefore, a mixture of radioactive and carrier selenite was employed in order to minimise the amount of arsenic in the incubation mixture.

EXPT. (2)

Determination of the rate of synthesis of dimethyl selenide by a rat liver cell-free system

Four weanling, male Wistar rats were maintained on an adequate diet until their weight had increased to 200-250g. The animals were then killed and 33% liver homogenates in 0.25M sucrose + 10^{-4}M EDTA were prepared. The homogenates were centrifuged at 9000g for ten minutes at 4^{o} after which the supernatants were removed and kept ice-cold.

The basic procedure outlined above for dimethyl-selenide generation was followed using $Na_2\,^{75}SeO_3$ and Na_2SeO_3. Twenty minutes after the selenite was added to the incubation tube, 0.5ml of the reaction mixture was withdrawn into 2.5ml of 5M sodium hydroxide solution. The contents of the incubation tube were again sampled at forty and at sixty minutes. The reaction in the tube was then stopped in the usual manner, and ^{75}Se was determined in the three aliquots withdrawn from the tube and in the residual fluid in the reaction vial; the percentage ^{75}Se lost from the reaction fluid at 20, 40 and 60 minutes was calculated, after adjusting for volume differences. The results are given in Table 8-7 and they show that between 62.5 and 86% of the total ^{75}Se volatilized is lost during the first twenty minutes of incubation.

In view of this, the incubation time for subsequent experiments was reduced to thirty minutes.

TABLE 8-7

Experiment to determine the rate of dimethyl selenide synthesis by rat liver cell-free extracts

Liver 9000g supernatants from four adequately-fed adult rats were used to study the synthesis of dimethyl selenide by the method described in the text. Figures represent the percentage of ^{75}Se radioactivity lost at the times shown.

Each supernatant was tested in triplicate and the values given are mean ± S.D.

9000g SUPERNATANT	INCUBATION TIME (MINS)		
	20	40	60
1	52.5 ± 0.1	55.7 ± 2.0	61.7 ± 0.6
2	60.7 ± 3.1	65.1 ± 2.7	68.9 ± 3.6
3	43.9 ± 0.4	59.8 ± 7.2	64.3 ± 7.0
4	66.4 ± 2.7	67.9 ± 3.9	76.2 ± 2.3

Determination of the residual ^{75}Se in the reaction tube itself after the reaction had been completed (Expt. 2) revealed very high radioactivity counts which, when expressed as d.p.m. per ml. of residual reaction fluid, were considerably greater than the radioactivity found in aliquots of the fluid which were

withdrawn during the experiment. A possible explanation was thought to be that dimethyl selenide volatilized from the reaction fluid, was not flushed from the incubation tube by the carrier gas but instead, became bound to the walls of the reaction vial (Fig. 8-2). This would lead to erroneously high residual radioactivity since both volatilized and non-volatilized ^{75}Se would be included in the residual radioactivity count. The next experiment was designed to investigate this possibility.

EXPT. (3)

Separation of volatilized and unreacted residual radioactive selenium

Two adequately fed adult male Wistar rats were killed and the 9000g supernatants of 15% liver homogenates were obtained. Each supernatant was tested in triplicate for dimethyl selenide generation activity, as described above.

The reaction was terminated after thirty minutes and the radioactivity in each tube was determined. The reaction fluid was then withdrawn into a clean tube using a fresh 1ml disposable syringe which was washed out afterwards with the 0.25M phosphate buffer used in the incubation mixture. The ^{75}Se count of the reaction fluid and of the washings of the syringe was determined. The empty reaction tube was also counted for any ^{75}Se which might have adhered to the walls of the tube. The results of this experiment are shown in Table 8-8.

TABLE 8 - 8

ANALYSIS OF THE RESIDUAL SELENIUM IN REACTION VIALS

9000g supernatants from two adult rats were used in dimethyl selenide synthesis experiments. The reaction was stopped after 30 minutes and ^{75}Se radioactivity in the tube was determined (Column A). The reaction fluid was then withdrawn and separate counts were obtained of the fluid (Column B) and the empty tube (Column C). The fourth and fifth columns compare the percentage losses obtained by using the different values of residual counts while the last column shows the percent of 'residual' ^{75}Se which has already been volatilised.

The values given are mean ± S.D. of three determinations.

	A Total residual radioactivity in fluid and tube (B+C) (c.p.m. x 10^{-3})	B Radioactivity in residual fluid only (c.p.m. x 10^{-3})	C Radioactivity in tube only (c.p.m x 10^{-3})	Percent ^{75}Se lost from tube i.e. $100-\left(\frac{A}{\text{initial count}} \times 100\right)$	Percent ^{75}Se volatilised from fluid. i.e. $100-\left(\frac{B}{\text{initial count}} \times 100\right)$	Percent of total residual ^{75}Se adhering to tube $\frac{C}{A} \times 100$
(1)	27.5 ± 1.2	16.3 ± 0.5	11.9 ± 1.1	15.0 ± 3.1	49.7 ± 1.2	43.1 ± 2.5
(2)	25.9 ± 0.6	15.4 ± 0.6	10.6 ± 0.2	19.6 ± 2.2	52.3 ± 1.7	41.0 ± 1.5

A comparison of columns 4 and 5 gives an indication of the size of the error which was obtained when the total residual ^{75}Se in both fluid and tube was used as opposed to ^{75}Se in the fluid only; the latter led to a more accurate and representative figure for the percentage loss of ^{75}Se from the incubation medium itself.

The last column in Table 8-8 gives the proportion of the total residual radioactivity which was not actually in the residual fluid. Diplock et al (1973) showed that dimethyl selenide can be quantitatively trapped in 8M nitric acid; this solution was therefore used to wash out the empty reaction tube in order that dimethyl selenide which had been deposited on the walls of the tubes may dissolve in the acid and thus be analysed. Table 8-9 shows the results obtained when the empty reaction tubes were washed with 2 x 1ml of 8M nitric acid; about 40% of the ^{75}Se that was previously classed as residual was in fact extraneous to the reaction fluid and, of this, 40-50% was dimethylselenide which had already been volatilized. The remainder was probably elemental selenium formed by oxidation of selenide. Therefore, to assess the activity of liver fractions in subsequent experiments, the incubation medium was withdrawn upon completion of the reaction and it was transferred to a clean tube for ^{75}Se determination.

TABLE 8-9

DEPOSITION OF VOLATILIZED DIMETHYL SELENIDE

Experimental details have been described in the text and in Table 8-8.

Values given are mean ± S.D. of three determinations.

9000g Supernatant from rats	C ^{75}Se deposited on tube walls (c.p.m x 10^{-3})	D ^{75}Se remaining in tube after 2 x 1ml washes with 8M nitric acid (c.p.m x 10^{-3})	E ^{75}Se which dissolved in 8M nitric acid (c.p.m x 10^{-3})	Percentage of the deposited ^{75}Se which was dimethylselenide $\left(\frac{E}{C} \times 100\right)$
(1)	11.9 ± 1.1	6.6 ± 0.8	5.3 ± 0.6	44.8 ± 3.4
(2)	10.6 ± 0.2	5.2 ± 0.1	5.4 ± 0.4	50.8 ± 2.4

F. Preliminary experiments on liver fractionation

Ganther (1966) used mouse liver extracts as the source of tissue enzymes to study the enzymic synthesis of dimethyl selenide. Various cell fractions were tested by him for the formation of dimethyl selenide and the specific activity of homogenates was found to increase as the nuclei, cellular debris and mitochondria were removed. His results are shown in Table 8-10 and specific activity was expressed as mμ moles of selenium volatilised per minute per mg. of protein x 10^{-2}

TABLE 8-10

SYNTHESIS OF DIMETHYL SELENIDE BY LIVER FRACTIONS

(GANTHER, 1966)

FRACTION	Protein (mg)	Specific Activity
0.3ml 10% homogenate	7.78	58
0.4ml 600g supernatant	8.80	62
0.5ml 9000g supernatant	8.22	73
1.0ml 100,000g supernatant	7.98	41
0.5ml microsome suspension	8.28	61
0.5ml washed microsomes	7.28	66
0.25ml washed microsomes +	3.64	
0.5ml 100,000g supernatant	3.99	78

Further centrifugation to separate the microsomal and soluble fractions resulted in a separation of activity. Washed microsomes were nearly as active as the 9000g supernatant; the soluble fraction had a low specific activity but it stimulated microsomal activity when the two fractions were recombined. Experiments using ^{14}C-labelled S-adenosyl-L-methionine and ^{75}Se-labelled sodium selenite (Ganther, 1966) showed that the initial rate of DMSe formation was low and that a lag phase of about five minutes preceded a fifteen-minute period of rapid synthesis. Therefore, measurement of the rate of DMSe synthesis during the first five minutes of reaction gave a value which was much lower (about 20%) than that obtained in a subsequent fifteen minute period. In a study

of the comparative rates of dimethyl selenide generation by various liver cell fractions, Ganther (Table 8-10) quoted the rates of DMSe synthesis by the subcellular fractions without indicating at which time during the incubation the rates were determined.

In addition, a further criticism of Ganther's work is that the unit of specific activity which was used to compare the liver fractions is not a suitable parameter for judging their relative activities in DMSe synthesis, since the calculation of these units was based on protein content of the liver fractions. The separation of cell fractions by differential centrifugation is as a result of the effect of centrifugal force on the size of the subcellular particle. Thus for example, the larger, more bulky fragments of plasma membrane which are present after homogenization, can be deposited in the pellet upon exposure of the homogenate to a low-speed centrifugal force of less than 1000g while the smaller ribosomes will remain in the supernatant even after centrifuging at 100,000g for one hour. Both of these cellular components contain protein and a comparison of their protein content is thought to be irrelevant to their activities in synthesising dimethyl selenide. Therefore, a more pertinent unit of specific activity was defined for the present experiments which served to express the activity of the subcellular fraction in terms of the weight of liver from which it was prepared. Thus, in the experiment described in the next section, the selenium volatilised by the

individual fractions was expressed in terms of the weight of liver in the homogenate from which the fraction was derived by differential centrifugation.

EXPT. (4)

Activity of Liver Subcellular Fractions in dimethyl selenide synthesis

One adult (330g), male Wistar rat was maintained on an adequate diet and was given supplemental selenium at 0.1ppm Se as sodium selenite in drinking water for the 24 hours prior to death. The additional selenium was administered in order to stimulate the activities of the liver enzymes involved in DMSe synthesis (Heinrich and Kelsey, 1955). Two 15% homogenates of the rat liver in 0.25M sucrose + 10^{-4}M EDTA solution were prepared (6gm tissue; 34mls solution). The homogenates were centrifuged, first at 9000g for ten minutes, after which the supernatant fractions (S_1) were decanted, and centrifuged again at 100,000g for one hour. The volumes of the 100,000g supernatant fractions (S_2) were measured and the microsomal pellets obtained were suspended in the same volume of homogenising medium, and re-centrifuged at 100,000g for a further hour (Fig. 8-3). The washed microsomes (Ms) were resuspended in their original volume, using a 3ml Potter-type tissue homogeniser. Experiments on dimethyl selenide generation were carried out using 0.3ml of the following in turn as the enzyme source:

(1) 9000g supernatant fraction (S_1)

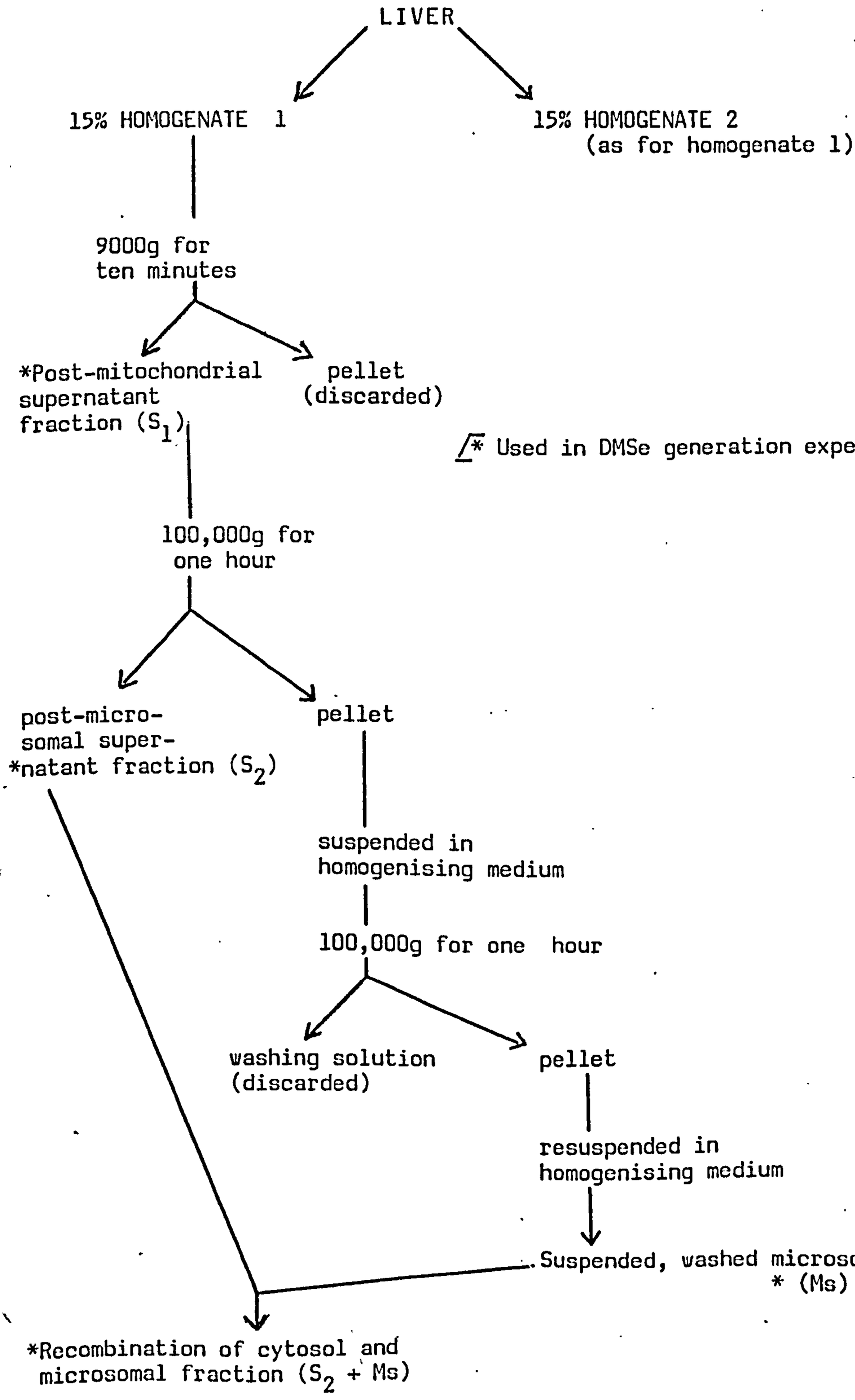
(2) 100,000g supernatant fraction (S_2)

(3) Washed microsomal fraction (Ms)

(4) Recombination of cytosol and washed microsomal fraction in equal proportions (5ml S_2 + 5ml microsomal suspension)

The post-microsomal supernatant fraction (S_2) and the suspension of washed microsomal fraction (Ms) were re-combined in equal volumes to produce a mixture which closely resembled S_1 in constitution. The ^{75}Se radioactivity in each experimental tube was determined both before and after the thirty minute reaction period. In addition, the protein content of each liver fraction was estimated using the colorimetric method of Lowry et al (1951) with an aqueous solution of bovine serum albumin (200μg/ml) as standard. The percentage of the initial ^{75}Se radio-activity which was lost from each tube was calculated. This was then expressed in terms of the protein content of the liver fraction used i.e. % ^{75}Se lost/mg protein. The weight of liver which was used in the preparation of each fraction was also calculated and the activity of the liver extracts was also expressed in these terms i.e. % ^{75}Se lost/g liver. All the results thus obtained are presented in Table 8-11. Comparison of the activities of the liver extracts shows that, when the results were expressed as the percentage of total ^{75}Se volatilised, the activities of the various fractions were as follows:-

Fig. 8-3. Preparation of Liver Fractions



/* Used in DMSe generation experiments/

TABLE 8-11

ACTIVITY OF LIVER FRACTIONS IN THE SYNTHESIS OF DIMETHYLSELENIDE

Post-mitochondrial (S_1) and post-microsomal (S_2) supernatant fractions and a suspension of washed microsomal fractions (Ms) were obtained from a 15% homogenate of rat liver by differential centrifugation - see text for details. These fractions, and a combination of S_2 and M_s, were tested in dimethylselenide generation experiments.

Total ^{75}Se volatilised from the incubation tubes was calculated (Column 1) and expressed in terms of the protein content of the fraction (Column 2) and the weight of liver used to prepare the fraction (Column 3).

Values given are mean ± S.D. of four determinations.

	(1)	(2)	(3)
LIVER FRACTIONS	PERCENT OF TOTAL ^{75}Se VOLATILIZED	% ^{75}Se VOLATILISED PER mg. PROTEIN IN THE FRACTION	% ^{75}Se VOLATILISED PER 0.1g LIVER FROM WHICH FRACTION WAS DERIVED.
9000g SUPERNATANT (S_1)	20.3 ± 6.8	4.6 ± 2.1	45.0 ± 3.7
100,000g SUPERNATANT (S_2)	38.7 ± 3.4	15.3 ± 5.7	49.6 ± 4.4
SUSPENSION OF WASHED MICROSOMES (Ms)	47.4 ± 4.6	36.7 ± 1.1	25.2 ± 2.5
MICROSOMES + 100,000g SUPERNATANT (S_2 + Ms)	44.9 ± 2.9	12.7 ± 0.9	26.1 ± 1.8

$$Ms \equiv (S_2 + Ms) > S_2 \gg S_1$$

When the results were expressed as the percentage of ^{75}Se volatilised per mg of protein in the fraction, this became:-

$$Ms \gg S_2 \equiv (S_2 + Ms) > S_1,$$

and when it was expressed as the percentage of ^{75}Se volatilised per 0.1g of liver, the results showed the following relationship:-

$$S_2 \equiv S_1 > (S_2 + Ms) \equiv Ms.$$

In this and other similar experiments, it became apparent that the washed microsomes only had a relatively high activity when the results were expressed in terms of the protein content of the fractions and it was felt that, since the liver extracts were separated by differential centrifugation which inevitably leads to an unequal distribution of protein between different layers in the centrifuge tube, without regard to which proteins are involved in selenium volatilisation, the protein content of the fractions should not be used as a reference on which to base calculations of their specific activity. In subsequent experiments therefore, the unit of activity used was defined as "the percentage of total selenium volatilised during the incubation period, per g liver." Thus the results in Table 8-11 would be interpreted as follows:- "the 9000g supernatant fraction obtained from a 15% homogenate containing 0.1g liver, volatilised 45 $\pm$ 3.7 per cent of the total ^{75}Se present in the

incubation tube." Where the liver extract used was a combination of microsomal fraction and 100,000g supernatant fraction, the interpretation would be:- "the microsomal fraction from 0.05g liver, when combined with the 100,000g supernatant fraction also obtained from 0.05g liver, volatilised 26.1 $\pm$ 1.8% of the total ^{75}Se present in an incubation tube."

Although the specific activities of the 9000g and 100,000g supernatant fractions (S_1 and S_2) were similar in value (Table 8-11), S_2 was preferentially used in experiments on dimethyl selenide generation in which the livers from animals on different dietary treatments were tested and only one subcellular fraction was analysed; this was because these experiments often also involved measurement of the activity of glutathione peroxidase (H_2O_2 : GSH oxidoreductase) and the 100,000g supernatant fraction was the only suitable fraction for the enzyme assays (see Chapter 9).

CHAPTER 9

GLUTATHIONE PEROXIDASE

(Hydrogen Peroxide: GSH oxidoreductase EC. 1.11.1. 9)

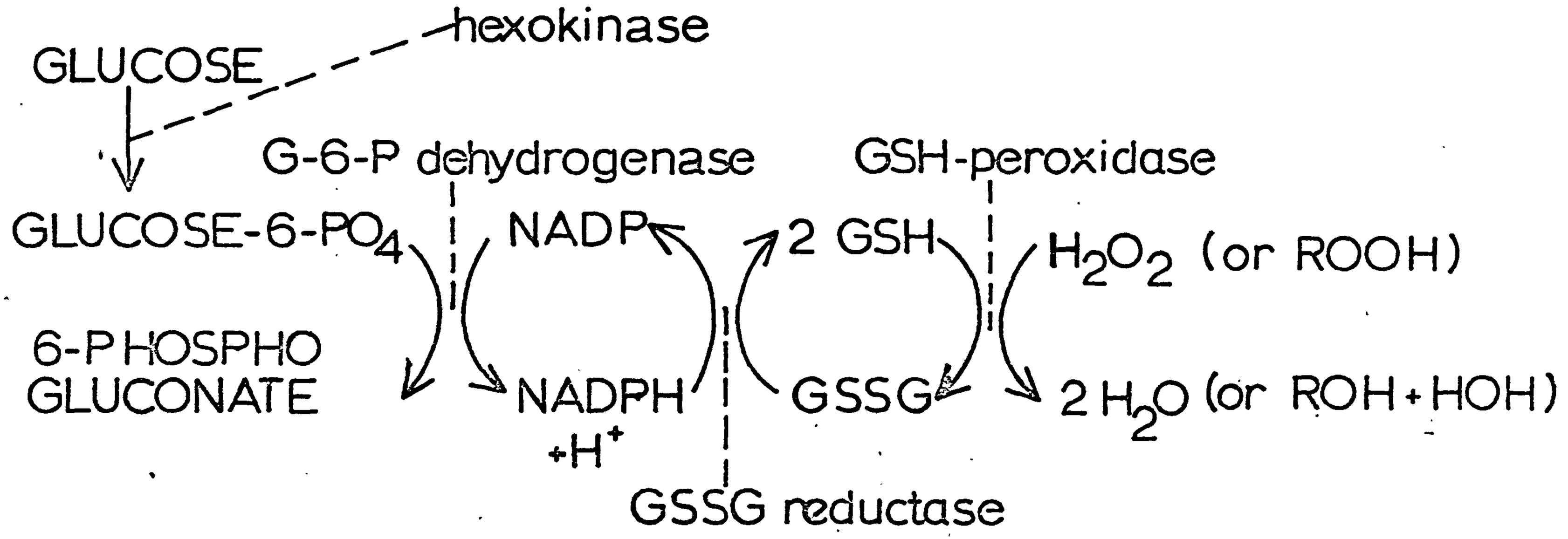
Glutathione Peroxidase (GSH-Px) was discovered by Mills (1957) who demonstrated the presence of a peroxidase in bovine erythrocytes that catalysed the breakdown of hydrogen peroxide, with glutathione serving as the hydrogen donor. Mills (1959) later purified the enzyme and confirmed that the peroxidase and catalase activities of the red cell could be attributed to two different enzymes. Cohen and Hochstein (1963) compared the role of erythrocyte GSH-Px and catalase in H_2O_2 destruction and concluded that under physiological conditions, the major pathway of H_2O_2 destruction involved glutathione peroxidase. Other workers (Little and O'Brien, 1968; O'Brien and Little, 1969; Christopherson , 1968, 1969) later established that GSH-Px also catalyzes the reduction of hydroperoxides formed from fatty acids or from other substances as well as hydrogen peroxide. The enzyme, therefore, catalyzes a general reaction which can be described by the equation ROOH + 2GSH $\rightarrow$ R-OH + HOH + GSSG(fig 9-1). It is apparent that glutathione peroxidase is widely employed in the protection of tissues against oxidative damage.

There have been many contributors to the pool of knowledge about the chemistry and biological function of GSH-Px. Among them have been Flohe and his co-workers in Germany who first succeeded in isolating weighable quantities of the pure enzyme (Flohe et al, 1971) and Flohe in the same year (1971) reviewed knowledge of the enzyme from 1957 to 1971.

At around the same time, Rotruck, Hoekstra and others at the University of Wisconsin, began an investigation of glutathione peroxidase in relation to the apparently inter-related nutritional roles of selenium, vitamin E and sulphur amino-acids. Hoekstra, in 1974, summarised the basis of their research into the biochemical function of selenium and the experiments that led to the discovery in 1973 that GSH-Px is a seleno-

Fig. 9-1

ENZYMIC ACTION OF GLUTATHIONE PEROXIDASE



protein. In addition, the biochemistry of GSH-Px has recently been reviewed by Ganther et al (1976).

The role of selenium in animal metabolism has been discussed further in Chapter 5 of this thesis.

Properties of Glutathione Peroxidase

A. Physical and Chemical Properties

Numerous estimates of the molecular weight of glutathione peroxidase have been made using gel filtration, sedimentation equilibrium or gel electrophoresis techniques. The purified bovine enzyme has a molecular weight of 84,000 in the erythrocyte (Schneider and Flohe, 1967; Flohe et al, 1971) while a somewhat greater value of 96,000 was estimated for the partially purified enzyme from bovine lens (Holmberg, 1968). Oh et al (1974) reported a value of 88,000 for the ovine erythrocyte enzyme while the human erythrocyte enzyme appears to be a larger molecule with a molecular weight initially recorded as 100,000 (Paglia and Valentine, 1967), but more recently shown to be around 95,000 (Awasthi et al, 1975).

Treatment of the bovine enzyme (Flohe et al, 1971) and of the ovine enzyme (Oh et al, 1974) with sodium dodecyl sulphate (SDS) and dithiothreitol, followed by acrylamide gel electrophoresis showed a single band of protein with an estimated molecular weight of 21,000 for the ovine enzyme; this protein was presumed to be one of four equal subunits of the native enzyme. Flohe et al (1971) further found that some degree of dissociation of the subunits takes place even in the absence of reducing agents, for example, upon electrophoresis in 8 M urea. They concluded that disulphide or other covalent bonds are probably not involved in linking the subunits together. The mobility of molecules under electrophoresis in SDS is primarily determined by the size of the polypeptide chain and not by intrinsic charge. No evidence for heterogeneity of the subunits in erythrocyte GSH-Px has been obtained. It would appear, therefore, that

the subunits have an identical size. However, this does not rule out the possibility of the subunits differing in their intrinsic charge by virtue of variations in their primary structure but the appearance of a single band upon electrophoresis in 8M urea makes this possibility unlikely. It thus appears that native GSH-Px consists of four identical subunits joined by noncovalent bonds.

Flohe (1969) determined the isoelectric point of bovine erythrocyte GSH-Px by zone electrophoresis, and obtained a value of 5.6 to 6.0 depending on the ionic strength of the buffer used; Oh et al (1974) obtained a value of 6 to 6.5 for the ovine enzyme, using the same technique. Spectral studies of the purified bovine enzyme (Flohe et al 1971 a) showed no absorption bands in the visible region, indicating the absence of haemes, flavins and other chromophores. The UV absorption spectrum shows a maximum at 280nm and an extinction coefficient of $E^{1\,cm}_{1\%} = 7.41$ (Flohe et al, 1971). From this, a molar extinction coefficient of 6.21×10^4 may be calculated. Studies on the ovine erythrocyte enzyme resulted in a molar extinction coefficient of $E^{1\,cm}_{1\%} = 7.06 \times 10^4$. An α- helix content of approximately 25% has been estimated for the bovine enzyme (Flohe et al, 1971) by circular dichroism studies.

Flohe et al (1973) first succeeded in crystalizing GSH-Px from bovine blood in 1.2M potassium phosphate buffer and the amino-acid composition of the enzyme has subsequently been determined by Nakamura and co-workers (1974). They reported that each subunit of the enzyme contains the following number of residues: Lys, 11; His, 4; Arg, 7; Tryp, 1; Asp, 14; Thr, 8; Ser, 9; Gln, 14; Pro, 10; Gly, 13; Ala, 10; Cys, 2; Val, 11; Met, 3; Ile, 8; Leu, 15; Tyr, 5; Phe, 7.

Fluorometric analysis of the 4000-fold purified enzyme showed a selenium content of 0.34% (Hoekstra et al, 1973; Oh et al, 1974) which is equivalent to approximately 4 gm-atoms of selenium per mole of enzyme.

This stoichiometry was confirmed by Flohe et al (1973) in a series of independent experiments involving activation analysis studies of the bovine enzyme. Since glutathione peroxidase is composed of four subunits which are similar in size, it is generally assumed that each subunit is associated with one selenium, although this has not been established. No other element was detected in the enzyme during the neutron activation analyses (Flohe et al, 1973).

The form of selenium in glutathione peroxidase has yet to be identified. It remains tightly bound to the enzyme during isolation but preliminary studies (Ganther et al, 1974) have indicated that much of the selenium is released from the enzyme in a low molecular weight fagment after long storage. A similar form of selenium was removed (Oh et al, 1974) from the ovine enzyme by ultrafiltration after the enzyme had been subjected to extremes of pH conditions. 50% of the selenium was filtrable after treatment with 40% NaOH or one N HCl for twenty-four hours at room temperature. The filtrate gave no fluorescent product with 2, 3-diaminonaphthalene even in the presence of a reducing agent, indicating that it was not selenite, or selenate, which can be reduced to selenite. Whether the selenium was released as a result of acid or base treatment, or after long storage, it had anionic properties which suggest that since it is not selenite, this form of selenium may represent a degradation product or a dissociable factor released during denaturation.

B. Substrate Specificity

The specificity of GSH-Px for glutathione is very high indeed, and few other compounds have been found capable of donating hydrogen for the reduction of hydrogen peroxide or other peroxides. Thus Mills (1959) found little activity with other tissue thiols, such as cysteine, cysteinyl-glycine and ergothioneine, when tested with the bovine erythrocyte enzyme. In addition, compounds that serve as donors for other peroxidases (such as o-toluidine, guaiacol or pyrogallol) also failed to show

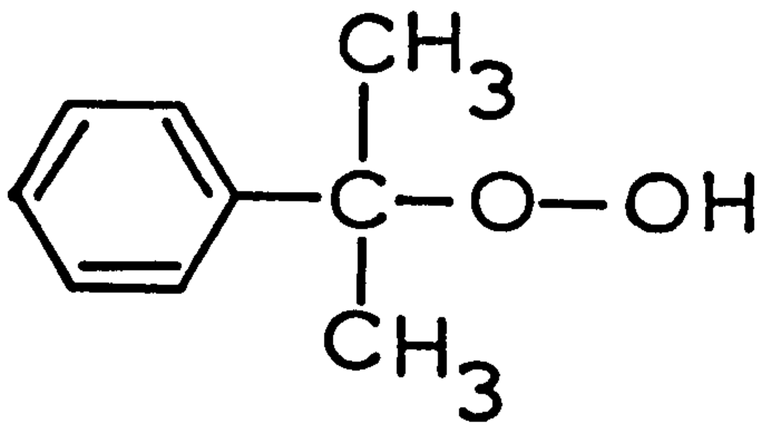
significant activity. In a study with twenty-nine different thiols, Flohe et al (1971 b) also concluded that glutathione is the only substrate showing significant activity. Only γ-L-glutamyl-L-cysteine methyl ester and mercaptoacetic acid methyl ester gave more than 10% of the activity obtained with glutathione. It was, however, of interest in this study that the possible importance of the α-carboxyl of the γ-glutamyl residue of glutathione in substrate orientation was indicated by greatly decreased enzymic rates with glutathione derivatives in which the carboxyl group had been esterified, or in which the γ- glutamyl residue was replaced by β-aspartyl or acetyl. Earlier studies (Flohe et al, 1970) with GSH-Px obtained from rat liver supernatant fractions revealed activity similar to that for the erythrocyte enzyme, when hydrogen peroxide was used as the other substrate. In contrast, Little and O'Brien (1968) who used cumene (C_9H_{12}) hydroperoxide and linoleic acid hydroperoxide as substrates for the liver enzyme, observed substantial activity for cysteine and cysteamine although these compounds were less active than glutathione.

When compared to its generally high specificity for glutathione, GSH-Px shows a rather low specificity for the peroxide substrate. In studies following the discovery of the enzyme, hydrogen peroxide was almost exclusively employed as substrate; however, Little and O'Brien (1968) have demonstrated that cumene hydroperoxide, linoleic acid hydroperoxide and tertiary butyl hydroperoxide (see fig 9-2) all serve as substrates for rat liver glutathione peroxidase. The enzyme from bovine lens has been shown (Holmberg, 1968) to behave in a similar manner, and Gunzler et al (1972) have described the kinetic behaviour of the bovine erythrocyte enzyme with various hydroperoxides; most of those tested were found to show some activity.

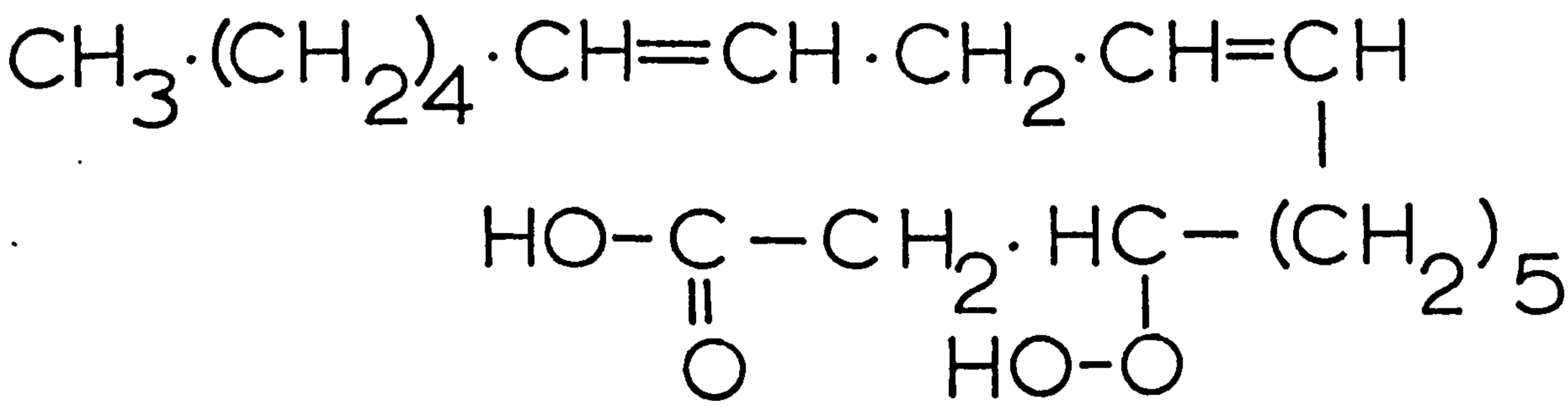
The list of hydroperoxides that serve as substrates has been extended by Little (1972) who tested several steroid hydroperoxides and

Fig. 9-2

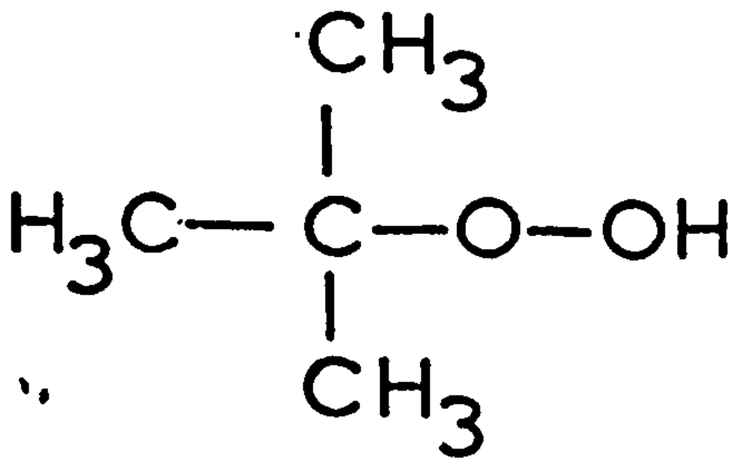
A. Cumene Hydroperoxide.



B. Linoleic acid hydroperoxide



C. Tertiary butyl hydroperoxide.



found progesterone 17 α- hydro-peroxide (fig 9-3) to be particularly effective. Steroid hydroperoxides are formed as intermediates in steroid hydroxylations and thus may be regarded as physiological compounds (Ganther et al, 1976). This, therefore, suggests a role for glutathione peroxidase which is beyond the protection of cell membranes from the lytic effects of oxidising compounds such as lipid hydroperoxides. The latter may also be regarded as physiological compounds in that they may be formed in sensitive membrane structures as a result of the spontaneous interaction of the poly-unsaturated fatty acyl moeities of membrane phospholipids and oxygen.

In addition to lipid - and steroid - hydroperoxides, "endoperoxides" produced as intermediates in the biosynthesis of prostaglandin PGE_2 and PGF_{2a} from arachidonic acid have been proposed (Ganther et al, 1976) as possible substrates of the glutathione peroxidase. This group of compounds (Hamberg et al, 1974) possess a peroxide bridge between ring carbons 9 and 11 (see fig 9-4), and one member of the group PGG_2 also has a C15 hydro-peroxy group. Another compound, (PGH_2) has a hydroxy group of carbon 15. The possibility that GSH-Px may catalyse reduction of the -OOH group in PGG_2 to -OH (as in PGH_2) or that the oxygen bridge may itself be attacked by the enzyme, has not yet been investigated.

C. Inhibition

Unlike other peroxidases, neither erythrocyte glutathione peroxidase (Mills, 1957; Mills and Randall, 1958) nor the liver enzyme (Little and O'Brien, 1968) is inhibited by cyanide or azide. These substances can, therefore, be added to assay systems in order to suppress catalase activity. In a test of various classis thio and dithiol reagents, Little and O'Brien (1968) found that 3mM p-mercuribenzoate or N-ethylmaleimide caused 60-70% inhibition while the same concentration of iodoacetate, iodoacetamide, cadmium chloride and sodium arsenite did not. Other workers (Schneider and Flohe, 1967) have determined that upon prior reduction

Fig. 9-3

Progesterone 17α-hydroperoxide

GSH-Px substrate

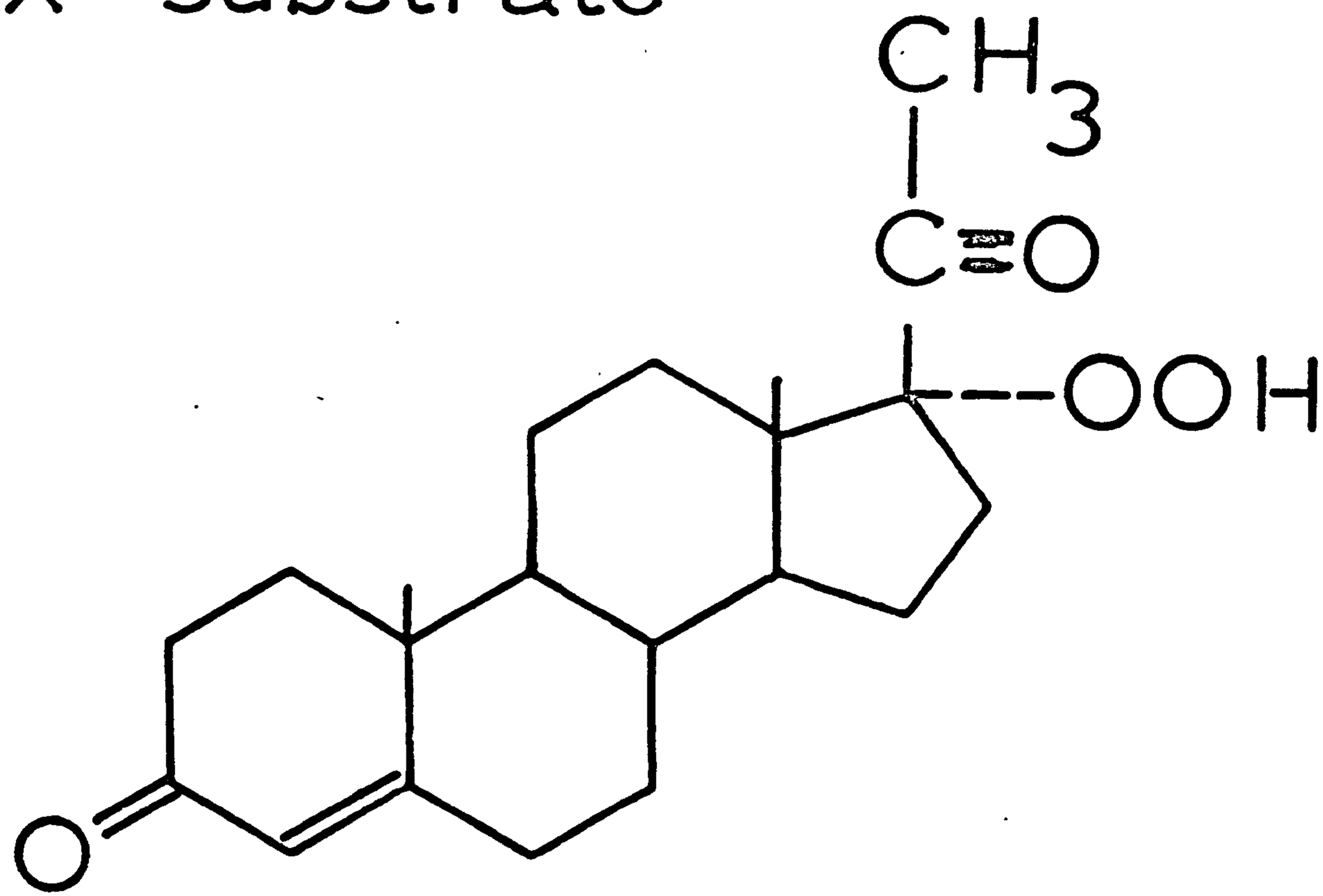
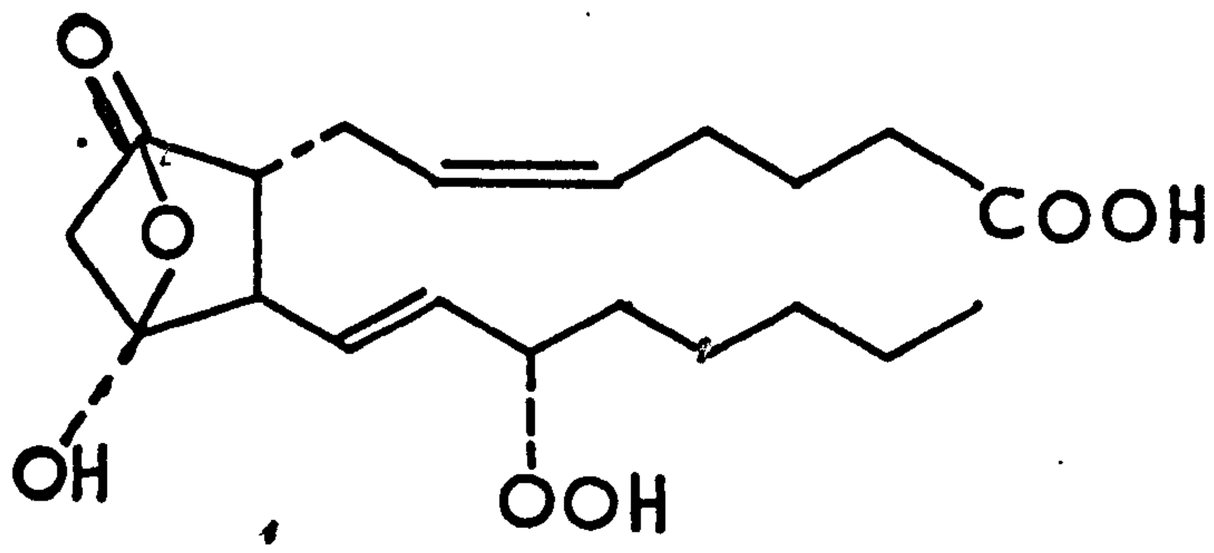
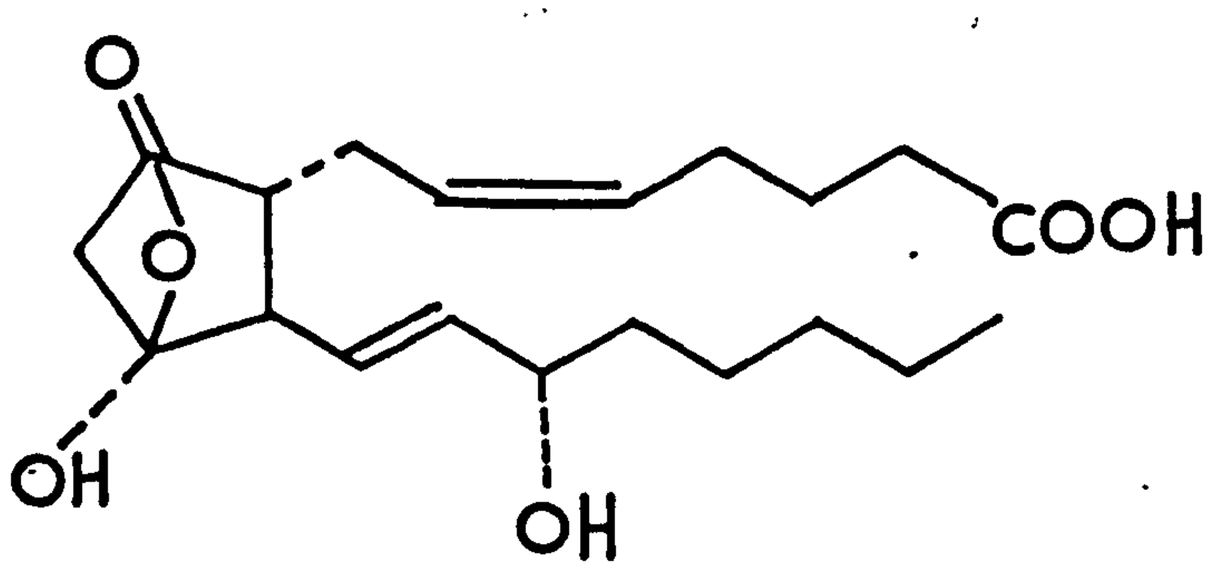


Fig 9-4. Endoperoxides —
Possible substrates of GSH-Px



PGG_2

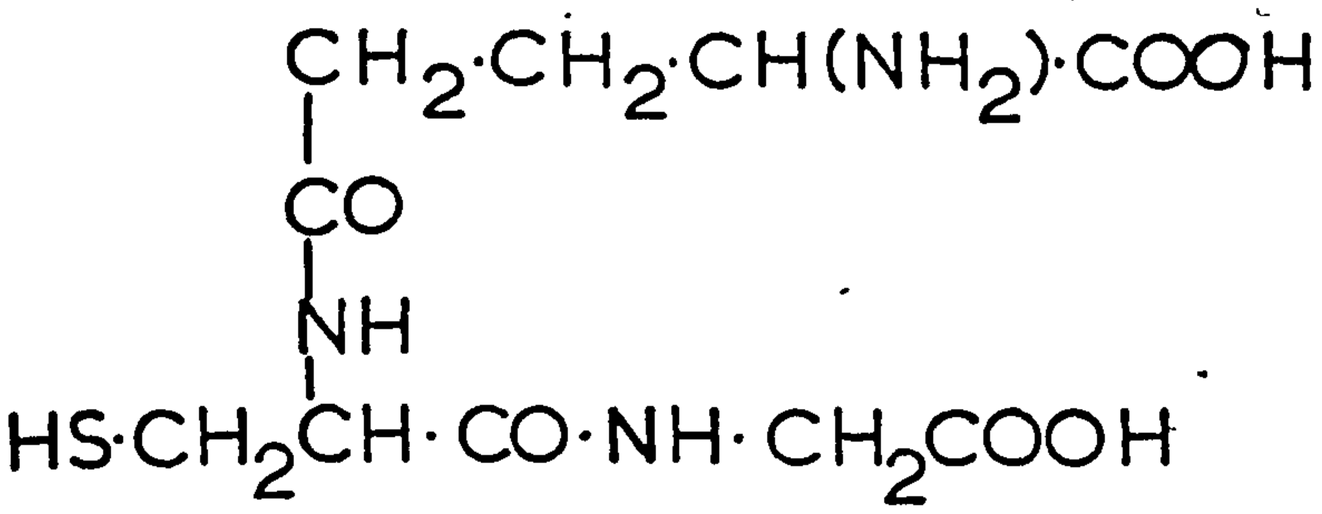


PGH_2

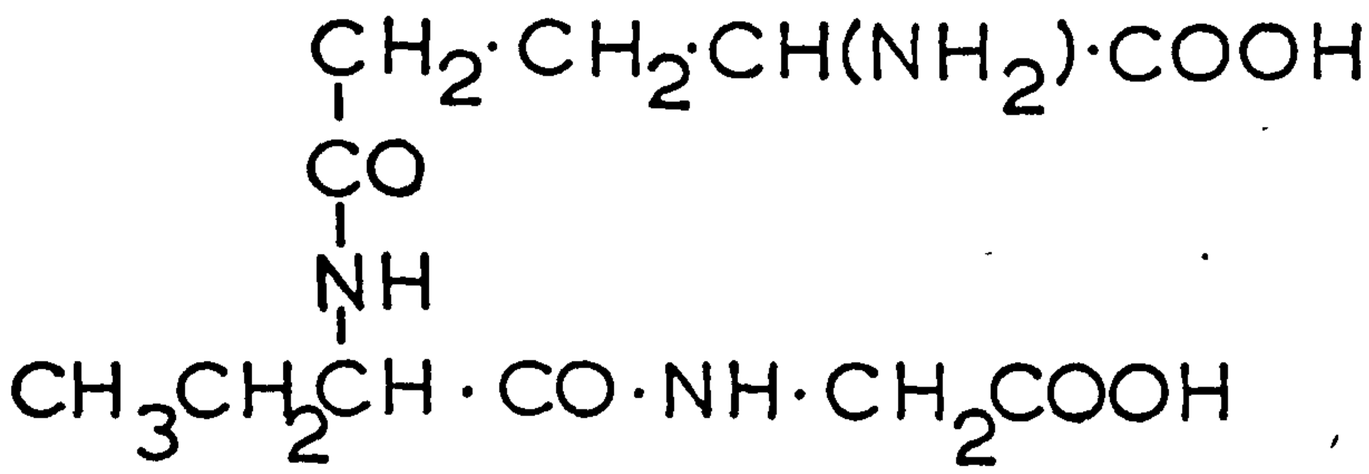
of the erythrocyte enzyme with 10^{-4}M glutathione, 2 x 10^{-4}M mercuric chloride was weakly, and reversibly, inhibitory. In the presence of ethylenediaminetetraacetic acid (EDTA) however, the same amount of mercuric chloride caused more than 90% inhibition. These results were interpreted by Schneider and Flohe (1967) as suggesting the formation of a complex between EDTA and mercuric chloride, and that the complex was then inhibitory to the enzyme. Substances bearing structural resemblance to the glutathione substrate were also tested as inhibitors: opthalmic acid (fig 9-5), which has a methyl group in place of the sulphydryl group of glutathione, was obtained from bovine lens and tested (Holmberg, 1968). It was not inhibitory to GSH-Px obtained from lens, nor did it affect the activity of the erythrocyte enzyme even when added in large excess (Flohe et al, 1971 b).

A number of substances unrelated to glutathione, except in anionic character are known to inhibit GSH-Px. High concentrations of multivalent anions, including phosphate, sulphate and maleate, caused reversible inhibition of GSH-Px, (Flohe and Brand, 1970). In addition, the effects of certain nucleotides on GSH-Px activity have been described. Little et al (1970) found a wide range of nucleotides, including NADPH (fig 9-6) to be more inhibitory than the phosphate anion. The inhibitory effect increased with the number of phosphate groups in the nucleotide and differential loss of catalytic activity and nucleotide sensitivity were observed after X-ray, ethanol or trypsin treatments. These workers later (Little et al, 1970 a) showed that coenzyme A (fig 9-6) is a much more potent inhibitor than other nucleotides, causing 50% inhibition at a concentration of 6 x 10^{-5}M . Although most of this effect was related to the nucleotide residue of CoA, some decreased effect was observed when the sulphydryl group was blocked. Erythrocyte levels of coenzyme A are sufficiently low to preclude a physiologically significant inhibitory action; in liver, however, it is possible that

Fig 9-5 Structural similarity of glutathione and opthalmic acid

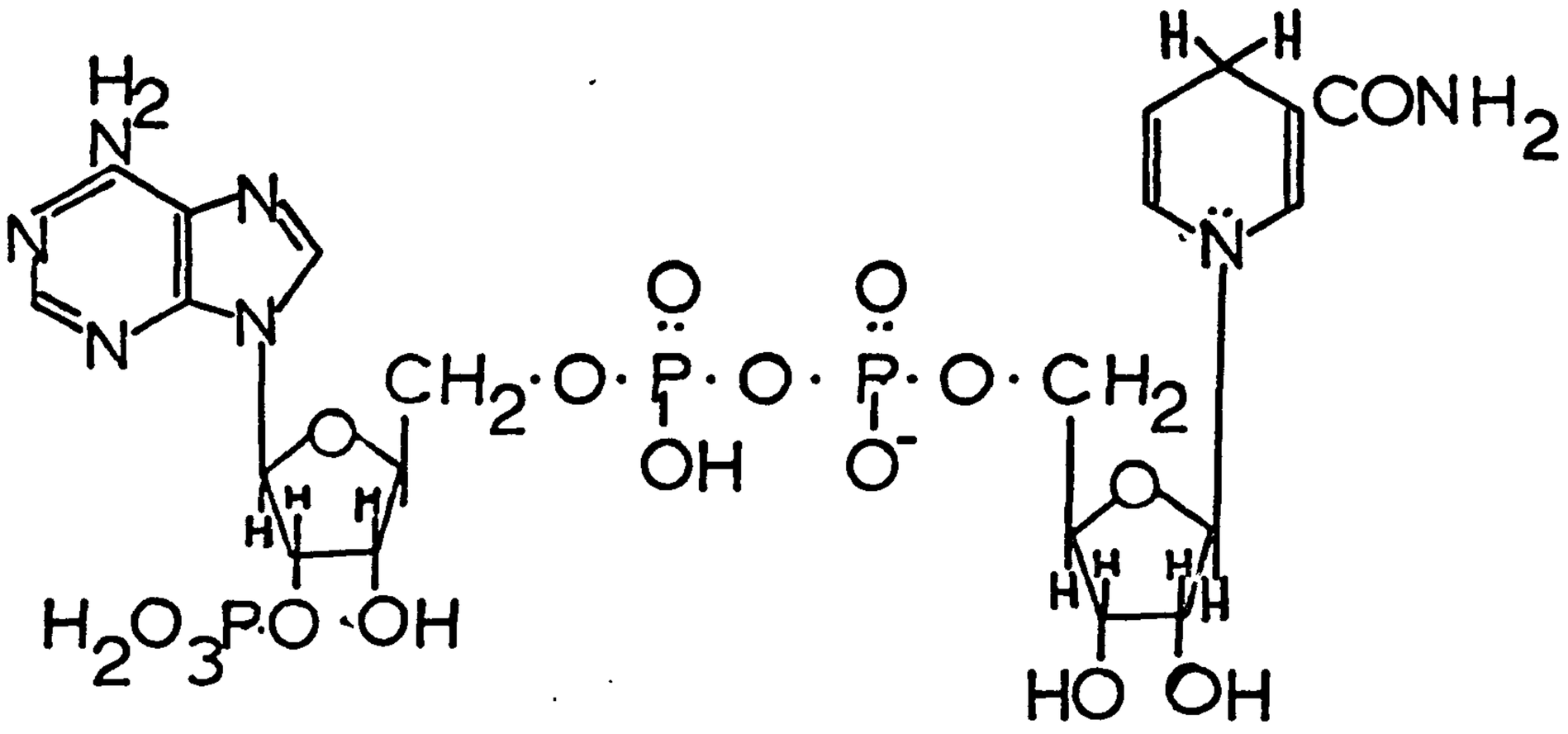


GLUTATHIONE (glutamyl cysteinyl glycine)



OPTHALMIC ACID (γ-glutamyl-α-, n-butyryl glycine)

Fig 9-6 NADPH



Nicotinamide Adenine Dinucleotide Phosphate (Reduced)

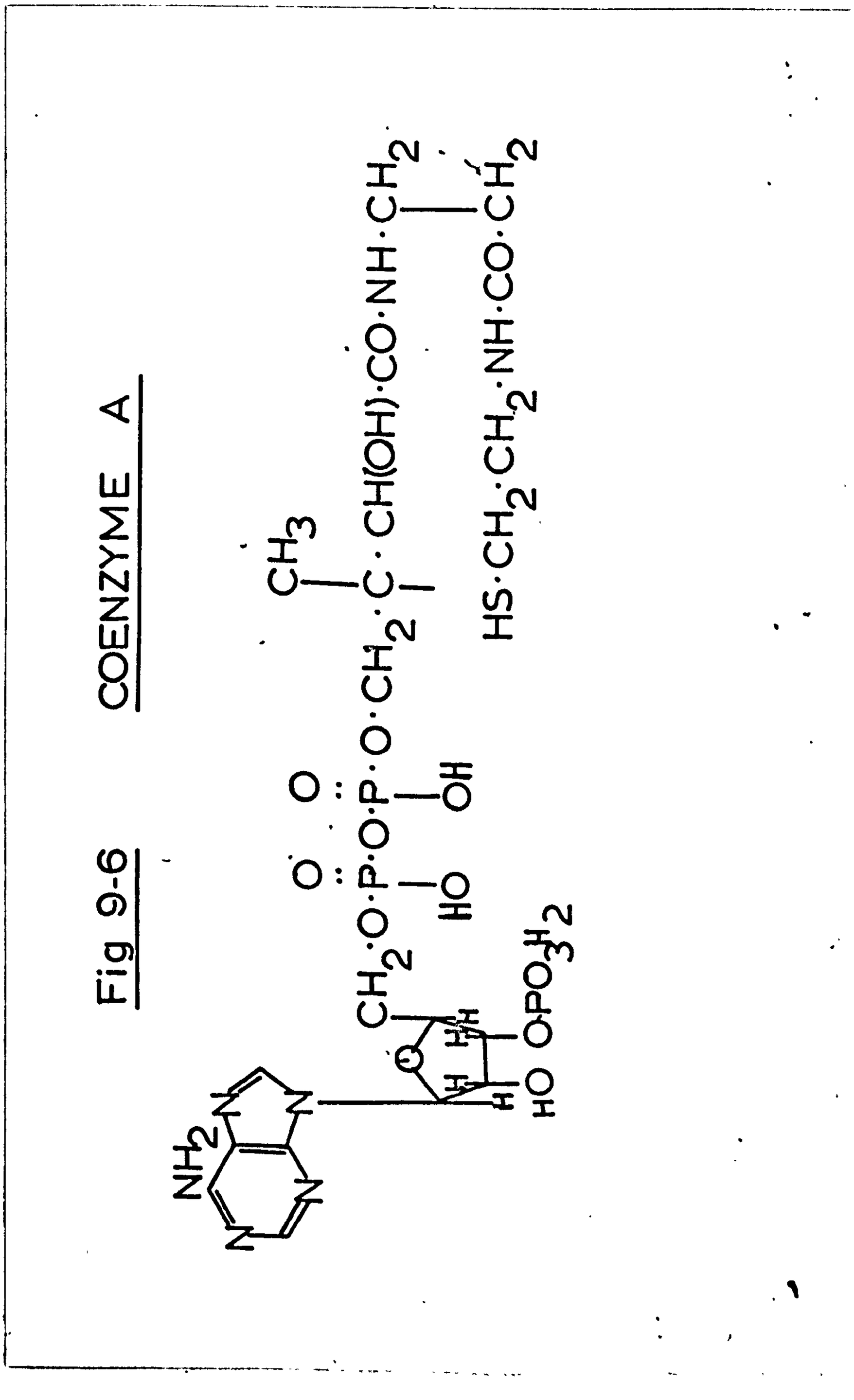

COENZYME A
CH2 CH2
CH3
HS·CH2·CH2·NH·CO·CH2·CH2·NH·CO·CH(OH)·C·CH2·O·P·O·P·O·
O: O:
OH HO
HO OPO3H2
NH2

Fig 9-6

coenzyme A is used to control glutathione peroxidase activity.

D. Kinetics

The kinetics of glutathione peroxidase catalysis are very complicated as has been shown by Flohe et al (1972) in studies using rapid reaction stopped - flow techniques. The general rate equation, although involving three molecules of substrate (ROOH, 2 x GSH), reduces to a fairly simple expression as many of the terms equal or approach zero. The maximum velocity and limiting Michaelis constants are indeterminate and the apparent K_m for H_2O_2 increases with increasing glutathione and has a value of 8.8μM at 2mM glutathione. The apparent K_m for glutathione varies between 10^{-7}M and 10^{-4}M when $[H_2O_2]$ = 10^{-9}M to 10^{-6}M (Flohe et al, 1972).

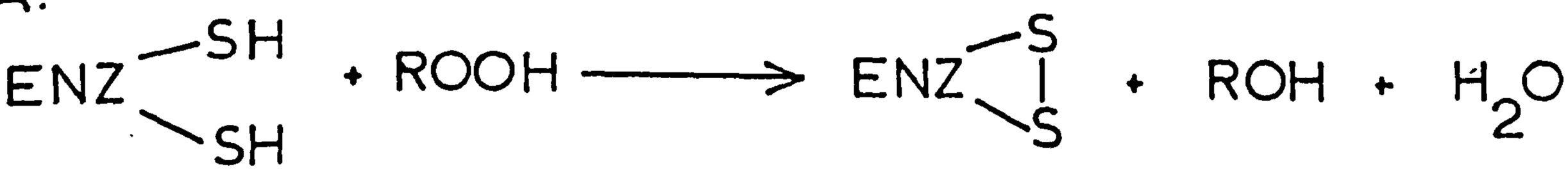
pH dependence experiments carried out by Mills (1959) and Holmberg (1968) showed that GSH - Px activity increases with decreasing acidity above pH 8. More detailed work by Flohe and others (1972) showed that enzymic activity is at a maximum at pH 8.8 and they concluded that denaturation was not responsible for the lower activities obtained below pH 10. Indeed, GSH - Px has no activity below pH 6 (Mills 1959), unlike many other peroxidases of plant or animal origin.

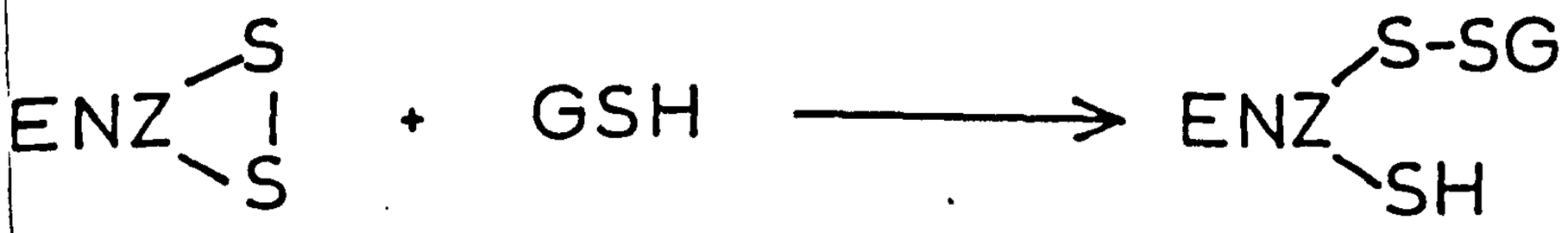
E. Enzyme Mechanism

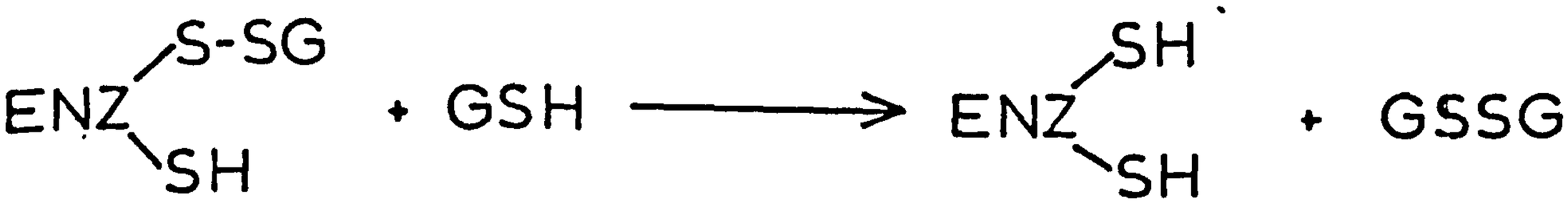
Prior to the discovery in 1973 that glutathione peroxidase is a selenoenzyme, two possible mechanisms had been proposed for its enzymic action. Both of these were based on the presence of a sulphydryl group at the active site, that formed a mixed disulphide intermediate with glutathione during the reaction. The first mechanism was proposed by Little et al (1970) and it involved the formation of ternary complexes between the enzyme, peroxide and glutathione. Flohe (1971) developed a more detailed model and proposed that the enzyme cycled between a dithiol form and a disulphide form (Fig 9 - 7A). Flohe rejected the

Fig. 9-7
Proposed mechanisms for glutathione peroxidase.

A. Flohe (1971)







B. Ganther et al (1974)

ENZ-SeH + ROOH ⟶ ENZ-Se-OH + ROH

ENZ-Se-OH + GSH ⟶ ENZ-Se-SG + H_2O

ENZ-Se-SG + GSH ⟶ ENZ-SeH + GSSG

mechanism of Little et al primarily on kinetic grounds and argued (Flohe et al, 1972) that the sequence he proposed of three bimolecular oxidation-reduction steps, seemed to be more consistent with the kinetic data. Further, participation of an active site disulphide could explain the finding (Flohe et al, 1971a) that treatment with sulphite caused reversible inhibition of the enzyme.

However, neither the mechanism of Flohe (1972) nor that proposed by Little et al (1970) was entirely satisfactory, and the failure of arsenite and cadmium to inhibit GSH - Px (Little and O'Brien, 1968) further weakened the case for a vicinal dithiol moiety. The argument of Flohe et al (1971a) that the thiol groups in the reduced enzyme might be too far apart for cadmium or arsenite bonding, ignores the fact that these -SH groups would have to be brought close together at some stage of catalysis to form the disulphide.

The discovery that GSH - Px contains 4 gm atoms of selenium per mole, provided an opportunity to consider afresh the mechanism of catalysis of the enzyme. Although selenium has not been shown to be present at the active site of the enzyme and thus to participate in catalysis, the chemical properties of the element and its presence in stoichiometric amounts with the number of subunits make it attractive to visualise its involvement. (Ganther et al, 1974). The proposed mechanism (Fig 9 - 7B) involves an active site selenol undergoing oxidation with peroxide to a selenenic acid (-SeH $\longrightarrow$ -Se-OH). In the second step, the oxidised form of the enzyme reacts with one molecule of glutathione, forming the mixed selenosulphide linkage (-Se-SG) which is attacked in the third step by a second molecule of glutathione. Oxidised glutathione is thus produced and the enzyme is returned to the selenol form. Circumstantial support for this mechanism can be obtained from different studies: the fact that three to four additional moles of p-mercuribenzoate became bound per mole of GSH-Px following reduction of the enzyme with glutathione

(Flohe et al, 1971a), can be attributed to the complexing of four selenol groups, one per subunit. Klayman (1973) has shown that selenenic acids, unlike sulphenic acids, are quite stable, especially if they are aromatic acids which are readily reduced to selenols by thiols. It is also known that the -S-Se- type of linkage is cleaved by thiols even more readily than the -S-S- linkage (Walter et al, 1969; Ganther and Corcoran, 1969).

Another possible set of reactions discussed by Ganther et al (1974) and analogous to those postulated by Flohe et al (1972), involved the interchange of selenium between selenenic (E-Se-OH) and seleninic ($E-SeO_2H$) forms and there is evidence to support the existence of both of these:- Little and O'Brien (1968) demonstrated the extreme sensitivity of the enzyme to N-ethyl-maleimide and p-mercuribenzoate, and this is more readily accounted for by a mechanism based on selenol. On the other hand, Chiu et al, (1977), in experiments using improved Xray photoelectron spectroscopy on rat liver glutathione peroxidase, obtained selenium electron signals which indicated that selenium in GSH-Px was not bound to oxygen. Further research is required in order to ascertain the chemistry of the enzyme in vivo.

DISTRIBUTION OF GLUTATHIONE PEROXIDASE

Although originally discovered in bovine erythrocytes by Mills (1957), GSH-Px has since been shown to occur in many organs, tissues, cells, subcellular components and fluids. Attempts to demonstrate its presence in plant tissues proved unsuccessful (Flohe, 1971) and no extensive study of its occurrence in micro-organisms has been reported. However, Grosch et al (1972) noted its presence in Candida lipolytica and Sunde and Hoekstra (1974) have obtained evidence of a non-specific, low catalytic activity peroxidase in Neurospora crassa.

In an early study, Mills (1960) compared the GSH-Px activity

of various tissues of the rat, and found liver to have the highest activity. Moderately high activity was observed in erythrocytes, heart, lung and kidneys while intestine and skelet al muscle showed slight and possibly insignificant activity. In recent surveys, the effect of dietary selenium status on the activity and distribution of GSH-Px in tissues of the rat and the chick was investigated. Differences in experimental procedure, assay conditions and definition of enzyme units have led to difficulties in comparing the results from one study to another. However, observations within a single study can be compared to give a reasonable indication of the relative enzyme activities of different tissues. In a review published in 1976, Ganther et al presented some of the more recent comparisons of GSH-Px activities of various tissues of animals that had received adequate quantities of selenium (at least 0.1 ppm Se) with those for animals given insufficient selenium (0.02 ppm Se or less). The data presented included determinations of GSH-Px activities by Hoekstra (1974), Hafeman et al (1974), Lawrence et al (1974), Chow and Tappel (1974) and Reddy and Tappel (1974) in tissues of the rat and by Noguchi et al (1973) and Omaye and Tappel (1974a) in tissues of the chick. In general, for rats given nutritionally adequate amounts of selenium, the body components can be divided into three categories on the basis of their glutathione peroxidase activity. Those tissues which have high GSH-Px activity include liver and erythrocytes: those with moderate activity include heart, kidney, lung, adrenal glands, stomach mucosa, pancreas and adipose tissue while low GSH-Px activity is found in brain, testis, eye lens and skeletal muscle. In the chick, the pattern is similar although the gross activity of the enzyme is lower. This is reflected in a comparison of results obtained from the same laboratory during the same period: - Chow and Tappel (1974), determined GSH-Px activities in rat tissues while the report by Omaye and Tappel (1974a)

concerned enzyme activities in chick tissues. GSH-Px activity in rat blood plasma was three times as high as that in chick plasma while the enzyme activity in liver was six times higher in rat than in chick.

In addition to the tissues mentioned above, GSH-Px activity has been found in phagocytic cells such as leucocytes and macrophages (Serfass et al, 1974) and also in blood platelets (Karpatkin and Weiss, 1972). Existing data on the blood plasma enzyme are somewhat confusing and the activities reported range from high to low values. It is therefore impossible to say whether the presence of the enzyme in plasma is due to leakage from tissues, platelets or other cellular components of blood, or whether the free enzyme exists in blood plasma in vivo and, if so, whether it has the possible functional significance proposed by Noguchi et al (1973).

Glutathione peroxidase activity varies with the dietary selenium intake of the animal, and this variation is also reflected in the relative body distribution of the enzyme. In studies of long-term selenium deficiency, some decrease in enzymic activity is observed in all tissues studied, but the magnitude of the decrease varies with the different tissues. In short term studies, plasma and liver GSH-Px are rapidly depleted on withdrawal of dietary selenium, while testis and brain enzyme levels are little affected; moderate decreases are observed in kidney, adrenal glands and erythrocytes. The smaller response of GSH-Px to dietary selenium intake in organs such as testis and brain may be due, in part, to a slow turnover of the enzyme in these tissues. In addition, there may be a mechanism which operates in conditions of limited dietary selenium, to allow the preferential utilization by some tissues.

The results of Tappel and his co-workers with the rat (Smith et al, 1974) and the chick (Omaye and Tappel, 1974a) have shown that the activity

of glutathione peroxidase increases in proportion to the logarithm of the dietary selenium concentration, and that this relationship exists even at chronically toxic levels of selenium. Hafeman et al (1974) observed a similar pattern for rat erythrocytes but found that liver showed a plateau of GSH-Px activity in the range of the selenium requirement, and a decrease in activity per unit liver weight (or liver protein) was observed when a chronically toxic dose of 5 ppm Se as sodium selenite was administered to the rats. Similarly, Oh et al (1974a) in studies with lambs, observed a continuous increase in erythrocytic and pancreatic GSH-Px activity with increasing dietary Se levels (up to 0.5 ppm). Eight other tissues assayed showed a plateau of GSH-Px activity at about 0.1 ppm selenium. Hafeman et al (1974) suggested that the continued increase of erythrocyte GSH-Px with increasing dietary selenium may be an adaptive method by which the erythrocyte can counteract the oxidant stress of excess selenite and that this adaption may be triggered in other tissues if the selenium intake exceeds a certain level. This suggests that the homeostatic regulation of selenium may be limited to a narrow range above the selenium requirement; however, further studies are needed before any pronouncements can be made on the relation between dietary selenium levels and glutathione peroxidase activity.

The subcellular localisation of GSH-Px in rat liver has been studied by Green and O'Brien (1970). They found that following the removal of nuclear and cell debris, 30% of the remaining activity was located in the mitochondria and 60% was in the soluble or cytosolic fraction. The lysosomal and microsomal fractions had a little activity which was attributed to contamination by mitochondrial fragments. Green and O'Brien (1970) also established that the matrix was the intramitochondrial site of GSH-Px localisation, and they tested the effect of various agents on the release of the enzyme from mitochondria.

They found that GSH-Px was released during swelling induced by GSH, GSH + GSSG, ascorbate and oleate and that the extent of the release was proportional to the degree of swelling. The enzyme was not released during the swelling induced by phosphate, Ca^{2+} or a mixture of phosphate + GSH. The conclusion was drawn that GSH - Px located within the mitochondrial matrix was responsible for protecting the inner membrane while the enzyme in the cytosol protects the outer membrane.

Recent studies of Levander et al (1974) have shown that a large proportion (at least 60%) of mitochondrial selenium is associated with glutathione peroxidase in livers of rats given 0.1ppm dietary selenium. In human erythrocytes, some GSH-Px activity has been found (Duchon and Collier, 1971) in the isolated membrane or 'ghost' fraction; however, it appears to be loosely bound.

In addition to selenium intake, other dietary, physiological, pathological and environmental factors have been shown to influence GSH-Px activity. Among these factors are the age and sex of the animal under investigation. Age and sex effects on rat liver GSH-Px have been demonstrated by Pinto and Bartley (1969a): they found that the enzyme activity of foetal male rat liver was about 35% of the adult male value, and there was a steady and rapid increase in liver GSH-Px from birth to 55 days, after which a plateau was reached. Aged, male rats (18 months) had a liver GSH-Px activity that was even higher than the young adult values - about 40% higher. Female rats had similar enzyme activities to the males at birth; by 45 days of age however, their liver GSH-Px had begun to rise above the male value and by 4 months of age, it was 80% above average male values. Still greater but qualitatively similar age and sex effects were observed by Demus-Oole and Swierczewski (1969) on rat liver soluble fraction. The sex difference was shown by Pinto

and Bartley (1969b) to be the result of increased liver GSH-Px caused by the female hormones oestrogen and progesterone, and to reflect a greater susceptibility of female liver to peroxidation *in vitro*, which was due in part to greater unsaturation of liver lipids. Although these sex effects have subsequently been confirmed by others (Little, 1972) they are apparently not manifested in other tissues, such as kidney and adrenal glands.

Another factor which influences GSH-Px activity is the presence of oxidant stressors, and recent evidence indicates that glutathione peroxidase is an adaptive enzyme often increasing in response to oxidant stressors. For example the exposure of rats to ozone (Chow and Tappel, 1972; 1973) led to an increase in lung cytosolic GSH-Px. Similarly, Reddy and Tappel (1974) have demonstrated an increased GSH-Px activity in several tissues of rats fed peroxidised corn oil in low-selenium diets. This effect was not observed when the Se content of the diet was adequate or more (2 ppm Se). In addition, Chow *et al* (1973) have demonstrated increased GSH-Px in vitamin E deficient rats, apparently caused by greater susceptibility of the tissues to peroxidation, due to the withdrawal of the antioxidant tocopherol.

It is of interest that animals with genetically-induced muscular dystrophy have increased GSH-Px activity in muscle tissue (Omaye and Tappel, 1974b), suggesting an increased susceptibility to peroxidation or possibly an increased production of oxidant stressors in the tissue.

A number of additional toxicants which cannot be classed as oxidant stressors or as antioxidants have also been shown to alter tissue GSH-Px activity. Some of these appear to exert their effect through an influence on selenium metabolism, particularly on its incorporation into glutathione peroxidase. Silver and tri-*o*-cresyl phosphate (TOCP) are examples of such toxicants. Bunyan *et al* (1968) have shown that

Silver precipitates the defects characteristic of selenium - vitamin E deficiency in rats and chicks. Other studies (Swanson et al 1974; Wagner et al, 1975;) of the effects of silver on GSH-Px, have demonstrated that the administration of 750 ppm silver (as silver acetate) to rats fed a high level of dietary selenium (0.5 ppm Se), produces a marked decrease in liver GSH-Px; lesser effects were observed in other tissues. Experiments similar to these are described in Chapters 14 - 16. In a systematic examination of silver stress in vitamin E-deficient rats and chicks, Diplock et al (1967) concluded that the physiology of the silver-induced liver necrosis in vitamin E-deficient rats was indistinguishable from that caused by selenium deficiency. This observation, together with the silver-induced decrease in GSH-Px, suggest that silver induces selenium deficiency by causing an almost complete block in the utilization of selenite for the ultimate synthesis of GSH-Px. The exact mechanism of reaction between silver and selenite (or its metabolites) is not known. It is known that rat tissues can reduce selenite to hydrogen selenide (see Chapter 8) and subsequent reaction with silver might lead to the formation of silver selenide. Further experiments concerning the role of vitamin E in the silver-selenium interaction, are discussed in Section III of this thesis.

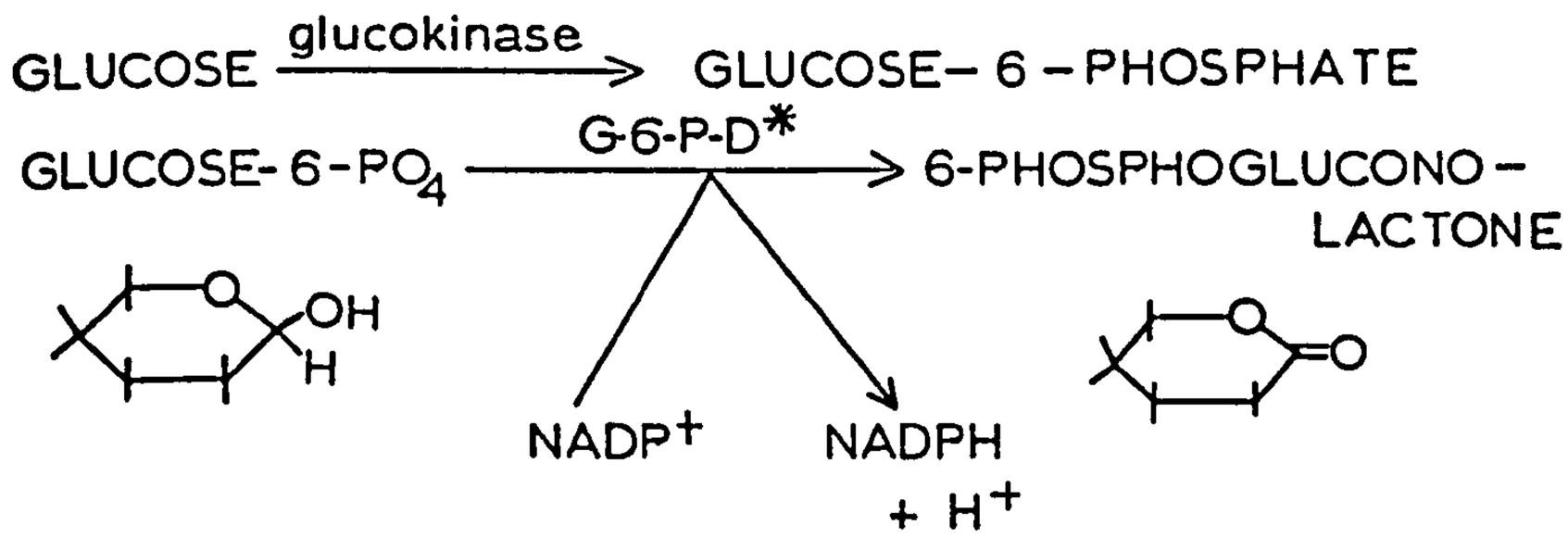
Tri-o-cresyl phosphate (TOCP) also produces toxicity effects in vitamin E-deficient rats. Diplock et al (1967) have shown that vitamin E alleviates these effects in rats and this finding has been confirmed and extended by Shull and Cheeke (1973) who showed selenium to be even more effective in protecting against TOCP-induced toxicity in rats and Japanese quail. TOCP produces similar effects to silver, resulting in decreased rat liver glutathione peroxidase activity and a smaller decrease in liver selenium content. The mechanism whereby TOCP, or its metabolites, interferes with selenium metabolism is not known but it may become clearer as a better understanding of the steps involved in selenium incorporation into GSH-Px is achieved.

(i) BLOOD: The role of glutathione peroxidase in the erythrocyte has been discussed by Flohe (1971) in a review of the enzymology and biological aspects of the enzyme; in addition, reviews on the more general topic of drug-induced hemolytic anaemias associated with enzyme deficiencies often include discussions of the function of GSH-Px in blood (Beutler 1972a, b; Jaffe, 1970; Kaplan, 1971).

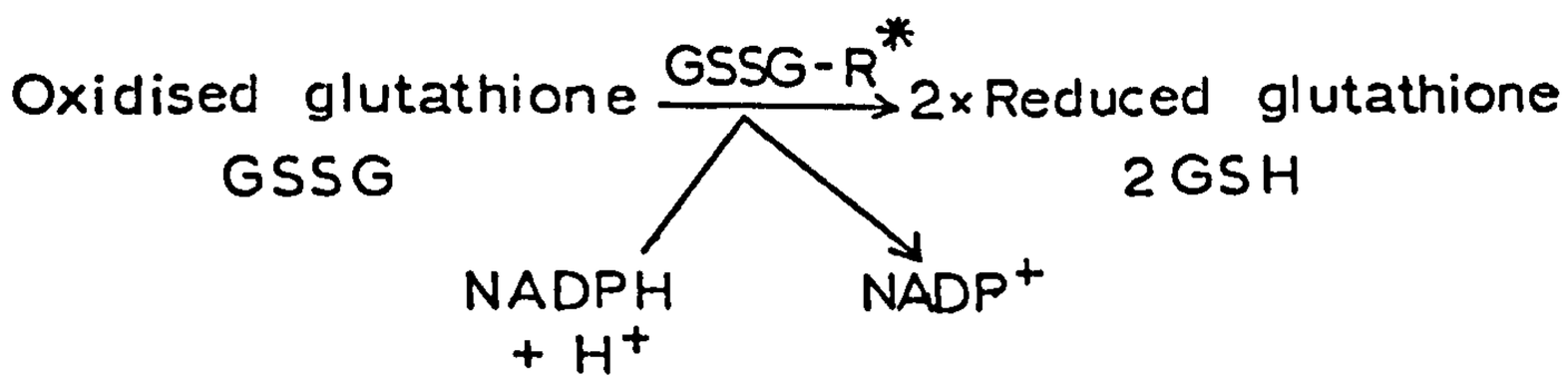
The protection of cells against oxidative damage by hydrogen peroxide involves the glutathione - dependent pathway shown in Fig 9 - 1. Hydrogen peroxide is formed in small but significant amounts in normal red cells during metabolic processes and is also generated within the cells as a result of the action of certain hemolysing drugs. The formation of H_2O_2 arises from the autoxidation of reduced flavins which catalyse the oxidative deamination of amino acids or are involved in the dehydrogenation of glucose-6-phosphate in erythrocytes. In addition, there are some enzymes such as xanthine oxidase and amino acid oxidases which yield hydrogen peroxide as an end-product. If not destroyed as rapidly as it is produced, H_2O_2 will oxidise haemo-globin to methaemoglobin and then, irreversibly, to oxidized degradation products; other substances essential for the integrity of the erythrocyte would then also be oxidised and haemolysis would follow. Fig 9 - 1 shows the mechanism whereby peroxides can be utilized and thereby removed from the red cell; glutathione peroxidase catalyses the preferential oxidation of glutathione by peroxides, and the subsequent reduction of GSSG by NADPH is catalysed by glutathione reductase (E.C. 1.6.4.2). As there is no electron transport system in the mature erythrocyte, there is no means of production for NADH (reduced nicotinamide adenine dinucleotide). However, the oxidation of glucose via the hexose monophosphate shunt (Fig 9 - 8) does ensure the maintenance of adequate levels of NADPH which can be used

Fig 9-8

THE PENTOSE PHOSPHATE PATHWAY



Reduction of glutathione



*G-6-P-D ≡ Glucose-6-phosphate dehydrogenase

*GSSG-R ≡ Glutathione reductase

in the reduction of GSSG. The hexose monophosphate shunt involves the phosphorylation of glucose and subsequent oxidation and hydrolysis accompanied by decarboxylation, resulting in the production of ribulose, a five-carbon sugar. Although glutathione peroxidase is the enzyme that actually participates in the removal of harmful peroxides, its efficiency is dependent on the activities of the other three enzymes involved in the protective pathway. Decreased activity of any of the enzymes in this pathway or of those involved in glutathione synthesis due to genetic alteration, the presence of inhibitors, dietary deficiencies or any other reason, would lead to impaired function of this pathway resulting in oxidative damage to haemoglobin and its precipitation into Heinz bodies with the eventual production of hemolytic anaemia. Several clinical cases of GSH-Px deficiency associated with hemolysis have been reported (Gharib et al, 1969; Necheles et al, 1970; Steinberg et al, 1970; Steinberg and Necheles, 1971;) and when the deficiency is severe, extensive hemolysis results even when no external oxidant stress is presented to the patient. It was at first thought that glutathione peroxidase deficiency may be due to genetic defect; however, the selenium status of the patient must now also be considered in cases with low GSH-Px activity.

The presence of relatively large amounts of catalase in erythrocytes, raises the question of the essentiality of glutathione peroxidase for the removal of hydrogen peroxide. Current opinion (Paniker and Iyer, 1969, 1972; Aebi et al 1964; Jacob et al, 1965; Nicholls, 1972) favours nearly equal roles for the two enzymes, with GSH-Px being more important at low concentrations of H_2O_2, where catalase is relatively inactive; conversely, at high concentrations of hydrogen peroxide, catalase is more important than glutathione peroxidase. However, in addition to catalyzing the destruction of hydrogen peroxide, GSH-Px catalyses the reduction of a wide variety

of hydroperoxides (Little and O'Brien, 1968; Little, 1972) against which catalase is inactive. The role of these other hydroperoxides in promoting the destruction of erythrocytes and haemoglobin is at present unknown. Cohen and Hochstein (1963) have shown that erythrocytes having normal levels of catalase but deficient in G6P-D are more susceptible to oxidant drugs than are normal erythrocytes; similarly, glutathione peroxidase-deficient erythrocytes (such as those obtained from Se-deficient animals) have shown (Flohe et al, 1972) an increased sensitivity to oxidative destruction. Flohe et al, (1972) concluded that this increased sensitivity is due, not to hydrogen peroxide-induced damage, which would be prevented by catalase, but to damage to the erythrocyte membrane caused by hydroperoxides of lipids that are produced by free-radical reactions (Pryor, 1973).

(ii) PHAGOCYTIC CELLS

The function of glutathione peroxidase in the protection of cells against oxidative damage from hydrogen peroxide and lipid hydroperoxides is of particular interest with regard to phagocytic cells. These cells include granulocytes (neutrophils, basophils, eosinophils) and monocytes in the blood as well as specialized tissue macrophages such as alveolar and peritoneal macrophages. Immediately following phagocytosis or the ingestion of foreign bodies (such as viruses and bacteria) there is a burst of respiratory activity in these cells and H_2O_2 is produced. This is followed by an increase in the activity of the hexose monophosphate shunt (Klebanoff, 1971). Although the ingested object is completely enclosed in a membranous sac (the phagolysosome), peroxides and other substances may diffuse from the phagolysosomes into the surrounding cytoplasm where enzymes such as catalase (Baehner, 1972), superoxide dismuthase (Fridovitch, 1974) and glutathione peroxidase (Flohe, 1971) can effect their conversion to non-toxic compounds. The presence of glutathione peroxidase has been demonstrated in a variety of phagocytic

cell types; among these are rat peritoneal polymorphonuclear leukocytes (Reed, 1969; Noseworthy and Karnovsky, 1972) rabbit alveolar macrophages (Vogt et al, 1971) and rat alveolar and peritoneal macrophages (Serfass et al, 1974). In addition, human peripheral polymorphonuclear leukocytes (Holmes et al, 1970; Noseworthy and Karnovsky, 1972) and guinea-pig peritoneal polymorphonuclear leukocytes (Strauss et al, 1969) are reported to contain moderate to low GSH-Px activity.

The coupling of GSH-Px activity to the hexose monophosphate (HMP) shunt results in the oxidation of NADPH to $NADP^+$ by glutathione reductase. The amount of NADPH produced is dependent on the amount of glucose utilized by the pathway; conversely, the rate of glucose oxidation by the H.M.P. shunt is controlled by the amount of NADP+ present (Beck, 1958). Thus, oxidation of glutathione by GSH-Px will cause oxidation of some NADPH to NADP+, and glucose oxidation will take place in order to restore the NADPH/NADP+ ratio to its original level. It is evident therefore, that glutathione peroxidase exerts a profound influence on glucose metabolism. The phagocytic cell responds to the ingestion of a foreign body by the production of large amounts of H_2O_2. Glutathione peroxidase catalyses the removal of the peroxide with subsequent production of GSSG which serves as a substrate for glutathione reductase. NADPH controls the activity of glutathione reductase and a fall in the amount of NADPH, due to its oxidation to NADP+, provides a stimulus to the hexose monophosphate shunt mechanism (Beck, 1958).

Thus, whereas normal levels of NADPH may suffice for the initial phases in the engulfing of foreign bodies, in order to maintain greater protective action, a stimulation of the hexose monophosphate shunt and GSH-Px activity may be needed. Therefore, in order for the cell to kill maximal numbers of bacteria before its lifespan ends, hexose monophosphate shunt stimulation may be vital to maintain glutathione levels, so that GSH-Px can provide sustained protection of the cell. Studies of polymorphonuclear leukocytes (PMNS) incubated with 10^{-4}M N-ethylmaleimide (Mandell, 1972) showed a decreased glutathione level and decreased protein

sulphydryl levels. The treated PMNS were able to function properly in the presence of low concentrations of bacteria, but N-ethylmaleimide interfered with phagocytosis when the bacteria:PMN ratio was higher (100:1). Stimulation of the hexose monophosphate shunt was impaired even though high levels of peroxide were generated. A study by Khandwala and Gee (1973) showed that 5×10^{-5}M linoleic acid hydroperoxide added to alveolar macrophages caused a 60% drop in bacteriocidal activity and a similar decrease in hexose monophosphate shunt activity. Glutathione peroxidase in the supernatant of the sonicated alveolar macrophages was inhibited 50% with 5×10^{-5}M N-ethylmaleimide. It thus seems likely that the effects observed upon treatment with N-ethylmaleimide, a glutathione antagonist, are due to GSH-Px inhibition; an impairment in the killing of yeast cells ingested by GSH-Px - deficient leukocytes has also been observed (Serfass and Ganther, 1975) although ingestion of the cells was not affected.

(iii) BLOOD PLASMA

The earliest reported study of glutathione peroxidase in blood plasma was that of Noguchi et al, (1973) who were investigating the possibility of a connection between glutathione peroxidase and exudative diathesis in the chick. This disease is prevented by selenium and vitamin E and is characterized by a severe oedema caused by the accumulation of a viscous fluid under the skin; Dam and Glavind (1940) showed that the permeability of peripheral capillaries was increased in exudative diathesis, with the result that fluid was exuded into the extra-capillary tissue spaces and the plasma was depleted of proteins, notably albumin. Noguchi et al (1973) measured GSH-Px in the plasma, erythrocytes and liver of chicks fed 0.1 ppm selenium as sodium selenite for 6 - 12 days after hatching; enzyme activities were compared with those of chicks fed the same diets minus selenium. In chicks given selenium in their diet, the specific activity of the enzyme in the plasma (measured in units per mg of nitrogen) was

comparable to that in erythrocytes and liver. When no selenium was administered, plasma GSH-Px had decreased to nearly zero after only 5 days of depletion, while the liver enzyme had decreased in activity by about 50% and the erythrocyte enzyme showed little change; the plasma enzyme fell to almost undetectable levels at least 2 days before there was any demonstrable incidence of exudative diathesis. The feeding of graded levels of dietary selenium, from 0 to 0.06 ppm showed that plasma GSH-Px activities were roughly proportional to dietary Se intake.

In a preliminary study, Smith et al (1974) showed that glutathione peroxidase activity was proportional to dietary selenium in some tissues; when rats were given selenomethionine in their diets to provide a level of selenium ranging from 0 to 2.0 ppm, there was a linear relationship between the logarithm of the selenium intake and the specific activity of the enzyme in all tissues studied. In a further study, Chow and Tappel (1974) examined the effects of feeding Torula-yeast based, selenium-and vitamin E-deficient diets to rats. The relative decrease in glutathione peroxidase activity was very rapid and extensive in plasma and was slow and of small magnitude in erythrocytes. Supplementation of the diet with 2 ppm selenium resulted in a significant linear increase in enzyme activity: that of the plasma enzyme was large and rapid while in erythrocytes, it was slow. Thus in rats, the largest decrease in enzyme activity in the absence of dietary selenium was observed in plasma (Smith et al, 1974) and the greatest response when selenium was re-fed was also in plasma (Chow and Tappel, 1974)

In a similar study in the chick, Omaye and Tappel (1974) observed that glutathione peroxidase activity in the plasma was strongly influenced by dietary selenium intake. The activity of the plasma enzyme of birds given 14 ppm Se was five times that of birds fed 0.1 ppm Se.

The relationship between enzyme activity in plasma and erythrocytes was unclear as the plasma GSH-Px varied between 60 and 100% of the erythrocyte enzyme.

The correlation between extremely low GSH-Px activities in chicks and the onset of exudative diathesis has been investigated by Scott and co-workers. A primary role for plasma glutathione peroxidase in preventing peroxidation of the plasma membrane of the capillary endothelial cells, was proposed by Noguchi et al (1973). They suggested that in the presence of nutritionally adequate levels of selenium, plasma GSH-Px is the first method of defence against peroxidation of the unsaturated lipids in the plasma membrane. Any lipid hydroperoxides either produced within the cell or which have managed to enter the cell, would be destroyed by cytosolic GSH-Px. Vitamin E, when present in adequate amounts in plasma membrane, would prevent the formation of such lipid hydroperoxides; in the absence of dietary vitamin E, Noguchi et al (1973) suggested that the hydroperoxides formed would be discharged into the lumen of the capillary endothelial cells, where they would be destroyed by plasma GSH-Px. When both selenium and vit. E are absent, no mechanism would then exist for the destruction of the peroxides, leading to oxidative attack on the plasma membranes which would result in an increase in their permeability, and hence explain the observations of Dam and Glavind (1940) of increased permeability of peripheral capillaries in exudative diathesis.

The absence of conclusive evidence on the plasma enzyme, such as isolation of GSH-Px from plasma, makes it difficult to establish whether or not apparent glutathione peroxidase activity is indeed due to the presence of the enzyme in plasma. In addition, in order to function, GSH-Px requires reduced glutathione and NADPH to keep it in the reduced state and there is very little NADPH and a limited supply of glutathione in the plasma. Srivastava and Beutler (1969) have reported the active

transport of glutathione from erythrocytes to plasma, in the form of GSSG, and the presence of glutathione reductase in significant amounts in plasma has been established by Chow and Tappel (1974). The presence and level of NADPH in plasma is therefore of great importance, since it determines whether or not GSH can be formed and thus, whether or not the hypothesis proposed by Noguchi et al (1973)is viable.

(iv) ACTION OF GLUTATHIONE PEROXIDASE IN RAT LIVER

Glutathione peroxidase is present in the liver of many species and, in some, the enzyme is present in rather large amounts (Mills, 1960). The fact that catalase is also present in the liver, has led to an investigation into the role played by GSH-Px in the destruction of hydrogen peroxide which is generated intracellularly. Hochstein and Utley (1968) demonstrated that GSH-Px and catalase compete equally for the small amounts of H_2O_2 which are generated by the action of glucose oxidase on glucose; in the presence of a high concentration of H_2O_2, the catalytic action of catalase was mainly responsible for its destruction.

It is now known that the major function of glutathione peroxidase may not be its role in H_2O_2 catabolism but rather its action in the decomposition of lipid hydroperoxides. While catalase can act only on low molecular weight organic peroxides such as methyl- and ethyl-hydroperoxides (O'Brien, 1969; Nichols and Schonbaum, 1963), GSH-Px reacts with many organic peroxides with little specificity.

Lipid peroxides are generated as a product of microsomal enzyme activity (Hochstein and Ernster, 1963) and although this activity is present in tissues such as muscle, brain and kidney, it is exceptionally high in liver (McCay et al, 1971). The system requires peroxide and NADPH with co-factors Fe^{2+} or Fe^{3+} complexed with adenosine di- or triphosphate or inorganic pyrophosphate (Hochstein et al, 1964; Poyer

and McCay, 1971). NADPH and oxygen are consumed and lipid peroxidation is accompanied by the release of malonyldialdehyde. The final result of the process is membrane fragmentation and dissociation of protein from the rough endoplasmic reticulum (Ernster and Nordenbrand, 1967; Hogberg et al, 1973). These physical alterations are reflected in changes in the activities of membrane-bound enzymes. Some enzymes, such as glucose-6-phosphate dehydrogenase, decrease in activity while others such as nucleotide diphosphatase and adenosine triphosphatase are activated. In general, these changes mirror those observed on treatment of microsomes with detergents (Ernster and Nordenbrand, 1967; Wills, 1971) suggesting that disruption of membrane integrity is responsible for the altered enzyme activities.

Lipid Peroxides:

Microsomal lipid peroxidation is inhibited by α-tocopherol, (Gram and Fouts, 1966), chelating agents (Ernster and Nordenbrand, 1967) and by antioxidants such as diphenyl-p-phenylenediamine (DPPD) (May and McCay, 1968). Glutathione in liver soluble fraction gives partial protection which is enhanced by the addition of the complete liver supernatant (Gram and Fouts, 1966). As discussed earlier in this chapter, liver glutathione peroxidase is predominantly a soluble enzyme although some activity is present in the mitochondria. Thus the presence of GSH-Px in cytosol together with glutathione and the enzymes necessary to maintain the level of reduced glutathione, may represent the major protective mechanism in liver supernatant fraction.

Ganther et al (1976) have suggested that the absence of, or a decrease in, glutathione peroxidase activity may be responsible for the necrotic liver degeneration observed in rats fed a Se-deficient diet low in vitamin E and sulphur aminoacids. The disease becomes fatal after feeding the diet for 3 - 4 weeks to weanling rats (Schwarz and Foltz, 1957),

and characteristic symptoms include degeneration of the endoplasmic reticulum and swelling of the mitochondria (Piccardo and Schwarz, 1958; Grasso et al, 1969). Hafeman et al (1974) have reported severe decreases in GSH-Px activity by up to 80% after only ten days of feeding the deficient diet to weanling rats; the enzyme was undetectable (< 1% of the control level) by the twenty-fourth day. Others have reported less severe, although highly significant, declines in rat liver GSH-Px caused by selenium deficiency (Smith et al, 1974a; Chow and Tappel, 1974; Reddy and Tappel, 1974).

The initial or latent phase of dietary liver necrosis in the rat is characterised by respiratory decline - an inability of liver slices, and homogenates to maintain normal respiration in vitro for extended periods (Schwarz, 1958). Either selenium or vitamin E prevents liver necrosis as well as respiratory decline in liver slices in vitro. In homogenates however, only vitamin E protects against respiratory decline (Schwarz, 1962) and the ineffectiveness of selenium may be due to dilution of cytosolic GSH-Px and glutathione in the homogenate medium. Glutathione, when present in adequate concentration, can protect in the absence of vitamin E or dietary selenium (Schwarz, 1962) presumably because of its non-enzymic reduction of hydrogen peroxide; Flohe (1971) showed that this reaction is appreciable at pH 7 or above and is also stimulated by high levels of GSH.

(v) GLUTATHIONE PEROXIDASE IN LENS TISSUE

The effects of peroxides and free radicals on lens tissue have not been investigated as thoroughly as in liver and blood. It does seem likely however, that similar manisfestations such as the oxidation of protein components and peroxidation of membrane lipids, both of which are thought to be involved in cataract formation, would be observed (Barber, 1973). Hydrogen peroxide is produced in the aqueous humour by

by oxidation of ascorbate (Pirie, 1965a, b) and in various parts of the eye by 1, 2 dihydroxynaphthalene (Rees and Pirie, 1967; van Heyningen and Pirie, 1967) a known cataractogenic agent. Pirie (1965b) has shown that H_2O_2 can diffuse into the lens in vitro, where the same glutathione-dependent pathway which exists in erythrocytes is presumably responsible for its removal (van Heynigen and Pirie, 1953; Pirie, 1965b). The existence of this pathway in lens would explain the importance of the very high levels of glutathione found in lens tissue and the same conditions that result in the precipitation of Heinz bodies and hemolysis in red cells, such as a decrease in activity of any of the enzymes involved in the maintenance of glutathione levels, might be expected to lead to cataract formation. In support of this, cataracts have been observed in patients with severe glucose-6-phosphate dehydrogenase deficiency (Westring and Pisciotta, 1966; Harley et al, 1966; Helge and Bainess, 1966).

GSH-Px activity has been demonstrated in lens tissue from various species and has been partially purified from bovine lens (Holmberg, 1968). This activity decreases with selenium deficiency (Lawrence et al, 1974) and cataracts have been observed in the second-and third-litter offspring of selenium-deficient rats (Sprinkler et al, 1971).

Catalase activity in the lens is low (Zeller, 1953) where it appears to be localised in the capsuk-epithelial area (Bhuyan and Bhuyan, 1970) and may be important in preventing H_2O_2 diffusion from the aqueous humor into the interior of the lens; however, it cannot prevent diffusion of H_2O_2 from other sources nor can it protect against other peroxides.

Cataractogenic drugs and chemicals may exert their influence by producing peroxides in amount too great to be detoxified by the GSH-Px protective mechanism, or by interfering with the operation of the mechanism. Naphthalene (Pirie, 1965b) and many of the anti-malarials (Bernstein, 1967; Cohen and Hochstein, 1967; Cohen and Hochstein, 1964) have been shown to

produce peroxides in the course of their metabolism and some cataractogenic chemicals probably produce free radicals or are metabolized to free radicals which can cause peroxidation of unsaturated lipids, nucleic acids and other compounds (Pirie et al, 1970; Kuck, 1970; Pryor, 1973).

In addition to selenium deficiency, several other essential nutrients have been implicated in cataract formation. Srivastava and Beutler (1970; 1972) have shown that riboflavin deficiency combined with other adverse factors can produce cataracts; this effect is probably a reflection on the role of riboflavin as a precursor of the cofactor in glutathione reductase. Vitamin E deficiency has also been shown to cause cataracts in turkey embryos (Ferguson et al, 1954; 1956) and vitamin E is effective in preventing the incidence of congenital cataracts in the litters of rats fed special cataractogenic diets.

Other effects of selenium mediated either via its role as a component of glutathione peroxidase or through other mechanisms, have been discussed in Chapter 6.

ASSAY OF GLUTATHIONE PEROXIDASE

There are three basic procedures by which the activity of glutathione peroxidase in various tissues may be measured, and choice of any one of these depends on the sensitivity required, the equipment available and the tissue under investigation. Flohe (1971) listed four main difficulties encountered in assaying the enzyme:

1. Non-enzymatic reaction of the substrates
2. Reaction of substrates with other physiological compounds
3. Inhibition of the enzyme by certain anions, and
4. Lack of a definite pH optimum.

In a later publication, (Flohe et al, 1972) reported a pH optimum for the enzyme, of 8.8 using organic buffers and rapid reaction techniques.

They also found, however, that the non-enzymic reaction increases with pH and is quite substantial at pH 8.8. Furthermore, the reaction mechanism is such that the limiting Michaelis constants and maximum velocities for both substrates are indeterminate; the apparent Km for each substrate is proportional to the concentration of the other so that it is not possible to saturate the enzyme with both substrates at the same time. Thus when the enzyme is assayed by direct measurement of product formed or the reactant remaining, the enzyme is not saturated with one of the substrates and the reaction is first order with respect to that substrate.

A. One of the three assay procedures for measuring glutathione peroxidase activity involves the use of glutathione as the limiting substrate: the reaction is stopped at intervals and the remaining glutathione is measured (Flohe, 1971, Mills, 1959). This procedure was first described by Mills (1959) and was later modified by Hafeman et al (1974) in order to determine enzyme activity in erythrocyte hemolyzates and liver homogenates. The assay medium containing 1.0 ml of 2mM GSH; 1.0 ml of 0.4M sodium phosphate buffer (pH 7), 0.4mM EDTA (to minimize the non-enzymic reaction); 0.5mlof 0.01M sodium azide (to inhibit catalase activity) and 0.03 ml of liver homogenate or erythrocyte hemolysate in a total volume of 4mlwas incubated at 37° for five minutes. 1.0ml of 1.25 mM H_2O_2 (also at 37°) was then added to start the reaction and 1.0 ml aliquots were removed at 3 minute intervals and added to 4.0ml metaphosphoric acid in order to precipitate the protein.

The glutathione content of the filtrate was determined (Mills, 1959) by converting it to the p-mercuribenzoate complex and obtaining the optical density of the sample at 255 nm. Hafeman et al (1974) added 5, 5' - dithiobis 2 - nitro benzoic acid to the filtrate and measured the optical density at 412 nm to determine the concentration of GSH.

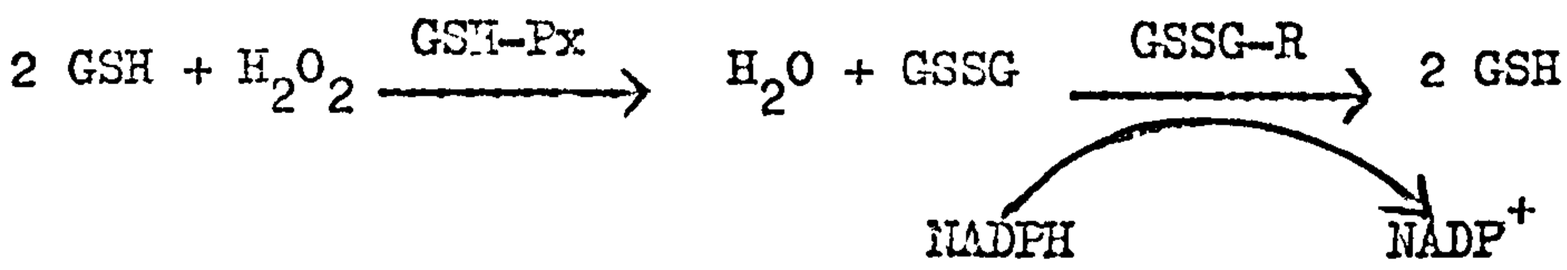
The non-enzymatic reaction rate was calculated by incubating a blank tube containing water instead of the enzyme source, simultaneously

with the samples and one enzyme unit of activity was defined as "the decrease in log [GSH] of 0.001 per minute after the decrease in log [GSH] per minute of the non-enzymatic reaction was subtracted."

B. Mills (1959) described another assay procedure for measuring GSH-Px activity in which the ability of the peroxidase - GSH system to prevent the oxidative breakdown of haemoglobin is utilized as a measure of peroxidase activity. Samples of crystalline rat oxyhemoglobin in isotonic Krebs-Ringer phosphate solution are incubated with glutathione, sodium azide, and ascorbic acid in saline solution at 37°. Peroxidase solution is added at zero time and the reaction is stopped after 90 minutes by treating the samples with carbon monoxide. The choleglobin content of the samples is determined and plotted against enzyme concentration. The amount of enzyme which produced a 50 per cent inhibition in choleglobin formation under these conditions was arbitrarily designated to represent one unit of enzyme activity. Mills (1959) suggested that this assay procedure could be used "for the qualitative assay of fractions from a column by using the percentage of inhibition of choleglobin formation as an indication of the presence of GSH peroxidase in the sample."

C. The third and most sensitive of the assay procedures was first developed by Paglia and Valentine (1967) for assay of glutathione peroxidase activity in red blood cells; since then, it has been adapted by different investigators for use in many different tissues. The method is similar to that described by Mills (1959) and later adapted by Hafeman et al (1974) in that it measures the rate of GSH oxidation by H_2O_2, as catalyzed by the glutathione peroxidase present in a hemolysate. Rather than measure the progressive loss of GSH, however, this substrate is maintained at a constant concentration by the addition of exogenous glutathione reductase (GSSG-R) and NADPH, which immediately convert any

GSSG produced to the reduced form:-



The rate of GSSG formation, and thus the rate of H_2O_2 reduction, is measured by following the decrease in absorbance of the reaction mixture at 340 nm, as NADPH is converted to NADP+. In this coupled assay, the GSH-Px reaction is made pseudo zero-order with respect to glutathione at saturating concentrations of peroxide. Since glutathione is continuously regenerated, its concentration does not decline during the reaction. Peroxide concentration does decline, but with the relatively high level of peroxide substrates employed by Paglia and Valentine (7.33×10^{-5}M H_2O_2 at a GSH concentration of 0.005M), the Km for peroxide (Flohe et al, 1972) is well below the peroxide concentration at all times. The enzyme is thus well saturated and the continuously recorded decline in A_{340} shows no indication that the reaction is slowing down until the NADPH which is needed for GSSG reduction, is nearly exhausted. A comparison by Emerson et al, (1972) of the coupled assay method of Paglia and Valentine with the direct method of Mills showed that the curve obtained when absorbance is plotted against time is linear during the initial phase of the coupled assay in contrast to the non-linearity of the same curve in the direct assay method. In addition, although Flohe and Brand (1969) have criticised the coupled assay method on the basis of their data showing catalysis of the reaction between hydrogen peroxide and glutathione by various haemoglobin derivatives, further investigation led them to conclude (Flohe and Brand, 1970) that conversion of haemoglobin to cyanmethaemoglobin as described by Paglia and Valentine (1967) by the addition of Drabkin's Reagent to the assay mixture lowers the rate of the reaction catalysed by haemoglobin considerably, and for a typical assay mixture, the cyanomethe-

moglobin-catalysed reaction is negligible.

Flohe and Brand (1969) have also shown that high concentrations of glutathione cause product inhibition of glutathione reductase and this fact makes the reaction unsuitable for Kinetic studies. However, routine assays of enzyme activity are not affected as excess glutathione reductase is used so that this enzyme is not rate-limiting.

THE GSH-PEROXIDASE ASSAY USED FOR THE PRESENT WORK

Preliminary experiments in our laboratory using post-microsomal supernatant of rat liver as the source of glutathione peroxidase, showed that a final peroxide concentration of 1.47×10^{-4}M in the cuvette gave an optimum rate of utilization of NADPH. This concentration is double that used by Paglia and Valentine. In addition, following the discovery of Little and O'Brien (1968) that peroxides other than hydrogen peroxide can serve as substrates for GSH-Px, cumene hydroperoxide was used in all the enzyme assays. The use of this substrate together with the addition of sodium azide to the assay mixture completely ruled out the possibility that catalase activity was being observed in addition to GSH-Px activity.

ASSAY PROCEDURE

The method used was basically that described by Paglia and Valentine (1967). Liver extracts were mixed with an equal volume of double-strength Drabkin's reagent (0.0016M KCN; 0.0012M $K_4Fe(CN)_6$; 0.0238M $NaHCO_3$) to convert all haemoglobin to the more stable cyanomethemoglobin form. 0.1ml of this mixture was placed in a 3ml silica cuvette and 2.58 ml of 0.05M phosphate buffer pH 7 containing 0.005M EDTA was added to the cuvette. The following solutions were also added to the mixture: 0.10 ml of 0.0084M NADPH: 0.01 ml Glutathione Reductase soln (= 0.01 i.u.); 0.01 ml of 1.125M sodium azide solution and 0.10 ml of 0.15M GSH solution. The mixture was

allowed to equilibrate at 20° in a unicam SP800 spectrophotometer and, after two minutes, the rate of reaction with endogenous peroxides was recorded by following the decline in the absorbance of the reaction mixture at 340 nm. 0.1ml of 0.0044 M cumene hydroperoxide solution was added to the cuvette, the contents were mixed and the conversion of NADPH to $NADP^+$ due to the newly added peroxide was then observed. The change in the rate of reaction, due to cumene hydroperoxide, was calculated to determine the true enzymatic activity. Enzyme units were defined as "the number of moles of NADPH oxidised per minute by one gram of liver" and were calculated on the basis of the molar absorbtivity of NADPH.

EXPERIMENT TO DETERMINE THE MOLAR EXTINCTION COEFFICIENT OF NADPH

Paglia and Valentine (1967) reported a value of 6.22×10^{-6} for the molar absorbtivity of NADPH. Glutathione peroxidase activities obtained by calculations based on this value were much lower than values reported by other investigators using similar assay procedures on rat liver extracts. An experiment was therefore designed to determine the molar extinction coefficient of NADPH.

For a typical GSH-Px assay, a stock solution of NADPH was freshly prepared on each experimental day and 0.1ml of this solution (equivalent to 0.0007g; 0.8 μmol NADPH) was used per assay. As the final volume of the assay mixture is 3 mls, the initial concentration of NADPH in the cuvette is 2.33×10^{-4} g / ml or 2.8×10^{-4} moles per litre. To determine the molar absorbtivity of NADPH, a solution containing 0.0050 g in 2 mls water was prepared. Aliquots of this stock solution were placed in a cuvette and diluted to 3 mls with 0.05M phosphate buffer pH 7.0 containing 0.005M EDTA. The spectrophotometer was set to zero with buffer and the absorbance at 340 nm of the NADPH solutions was read against the buffer (Table 9-1). A graph of absorbance against concentration of NADPH was plotted (Fig 9-9);

TABLE 9 - 1 DETERMINATION OF THE MOLAR ABSORBTIVITY OF NADPH

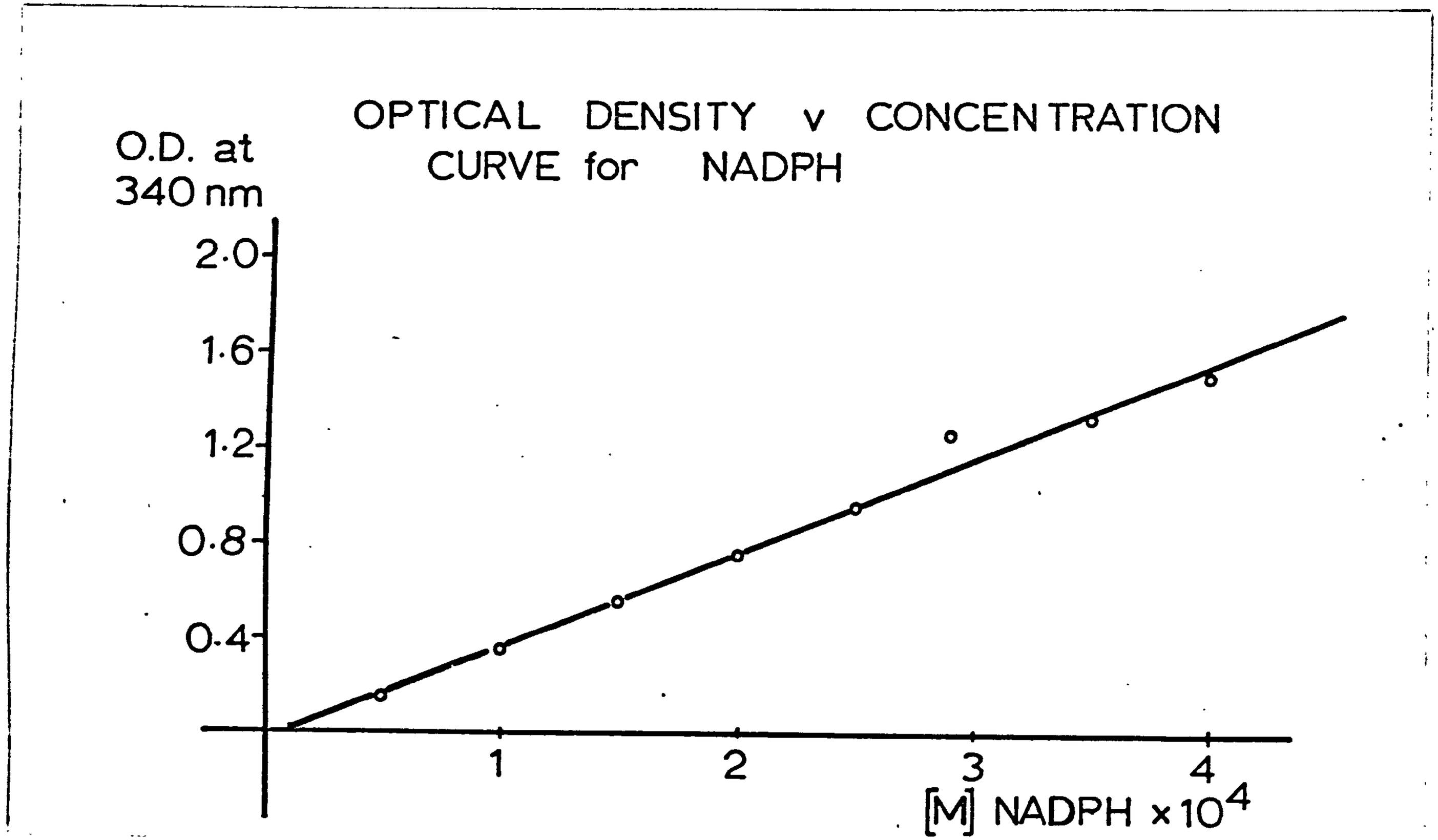
Aliquots of a stock solution (0.0025 g NADPH/ml) were diluted in a cuvette to 3 mls; O.D. at 340 nm was read in a unicam SP800 spectrophotometer.

Vol. stock solution taken for dilution to 3 mls	Wt. NADPH in aliquot	Final concentration in moles per litre	Absorbance at 340 n.m.
0.05 ml	.000125 g	.00005	0.16
0.10	.00025	.00010	0.36
0.15	.000375	.00015	0.56
0.2	.0005	.00020	0.76
0.25	.00063	.00025	0.96
0.3	.00075	.00029	1.26
0.35	.00088	.00035	1.33
0.4	.00100	.00040	1.51

Fig. 9-9.

Experiment to determine the molar extinction coefficient of NADPH.

Values used to plot the graph are given in Table 9-1. The slope and intercept of the line are recorded in the text.



the slope of the line was calculated to be 0.3974×10^4 and the intercept on the ordinate axis was 0.02. The equation of the line was thus

$$y = 0.3974 \times 10^4 x - 0.02$$

and this can be approximated to

$$y = 0.3974 \times 10^4 x$$

where the variable y is absorbance of NADPH at 340 nm and x is the concentration of NADPH in moles per litre. Thus a solution containing 1 mole NADPH in 1 litre should have an optical density of 3974 O.D.units.

[NADPH]	OPTICAL DENSITY
1 M	3974 units
2.516×10^{-4} M	1 unit

Thus, by measuring the change in O.D. of an assay mixture as NADPH is converted to NADP, the concentration of NADPH represented by the change in absorbance can be calculated and expressed in terms of enzyme activity.

PREPARATION OF LIVER EXTRACTS FOR USE IN GSH-Px ASSAYS

In general, liver fractions used in glutathione peroxidase assays recorded in this thesis were prepared by the techniques described in Chapter 8 for the separation of liver fractions by differential centrifugation of liver homogenates. By weighing the liver before it is homogenised and measuring the volumes of the homogenates and the supernatants obtained after centrifuging the homogenates, it is possible to calculate the amount of liver from which a sample of the fraction has been derived. For example, a 25% liver homogenate is prepared using 5 g liver tissue and the homogenate (20mls) is centrifuged at 9000 g for ten minutes; the volume of the 9000 g supernatantwas measured and found to be 18.5mls.

∴ 0.05mls of this supernatant which is used in each GSH - Px assay, represents the 9000 g supernatant which would be obtained from

$$\frac{5}{18.5} \times 0.05 \text{ g liver}$$

$$= \underline{0.0135} \text{ g}$$

Thus, the enzyme activity obtained from O.D. measurements and expressed as moles NADPH oxidised per minute, would be divided by this weight of liver (0.0135 g) to obtain the unit of enzyme activity viz. the number of moles NADPH oxidised per minute by the 9000 g supernatant from one gram of liver.

ADDENDUM TO CHAPTER NINE

During the late stages of the preparation of this thesis, an important publication by the group of Dr. Al Tappel of the University of California, came to hand. They (Forstrom et al, 1978) reported the identification of the selenium moiety in glutathione peroxidase as selenocysteine and described detailed analysing techniques involving the preparation of a derivative of the selenium moiety and cochromatography with known standards. The selenocysteine was identified as the catalytic site in the enzyme and it was concluded (Forstrom et al, 1978) that the reduced form of glutathione peroxidase contains the selenocysteine selenol (- SeH) at the catalytic site.

CHAPTER 10

SELENIUM DETECTION AND ESTIMATION

With the growing importance of selenium in Biochemistry and Chemistry as well as in industrial processes, the need becomes apparent for precise methods of analysis for the element. This is reflected by the large number of publications in the last fifteen years which deal with selenium in all its aspects. There are several methods of selenium analysis and choice of any particular one is dependent on a variety of factors, such as the source and nature of the selenium. For any method of analysis, the sample must be carefully prepared to enable easy and precise determination of the selenium. Preparation generally involves decomposition of the material and measurement of the selenium in the decomposition product; the latter stage may involve a separation step.

A. PRELIMINARY TREATMENT OF THE SAMPLE BY DECOMPOSITION

1. FUSION

One of the earliest used methods of decomposition and which is still in use today, is the fusion method in which the sample is fused with an inorganic material to destroy the organic matter (Alicino and Kowald, 1973). The most common types of fusion are:-

(a) carbonate fusion - where Na_2CO_3 + KNO_3 or Na_2CO_3 + $NaNO_3$ is the mixture used.

(b) alkali fusion with NaOH and

(c) peroxide fusion with sodium peroxide

The carbonate fusions are carried out in platinum crucibles, while the alkali and peroxide fusions require iron or nickel crucibles.

The selenium is usually oxidised to its hexavalent form and, after treatment with water, other elements such as boron, silicon, aluminium, tungsten and molybdenum are solubilized along with the selenate; metals such as iron, copper and nickel are deposited as insoluble oxides and

carbonates.

2. WET DIGESTION

This type of decomposition is also known as wet ashing or minerilization. McNulty (1947) described the digestion of materials such as soil, nutrient media or tissues on a large sample scale. He used ammonium metavanadate in nitric acid to digest the sample, followed by the addition of nitric, sulphuric and perchloric acids. Finally, a high temperature ($210^{o}C$) was applied to complete the digestion.

Kahane and Korach (1951), working with 5-50 mg of sample, added 1.5 ml of nitric acid and 2 ml of perchloric acid and utilized a water trap to absorb small amounts of selenium that might be vaporized.

In a comprehensive series of experiments, Gould (1951) digested organoselenium compounds by the addition of 3ml of concentrated sulphuric acid, followed by smaller portions of fuming nitric acid. He determined the selenium content of 22 organoselenium compounds and found that the level of Se in these compounds ranged from 10 to 30%.

B. SELECTIVE SEPARATION

1. VOLATILIZATION AND DISTILLATION

Gooch and Pierce (1896) used the reaction of selenite with hydrobromic acid to separate selenium from other materials:-

$$H_2SeO_3 + 4HBr \longrightarrow SeBr_4 + 3H_2O$$

The pollutants are left behind on distillation and the distillate contains selenium dibromide and bromine.

$$SeBr_2 + Br_2 \xrightarrow{3H_2O} H_2SeO_3 + 4HBr$$

After treatment with water, the selenium may be determined in a variety of ways.

McNulty et al (1951) used this technique to remove selenium from copper and organic materials and then determined selenium iodometrically after destroying the bromine. This method was also used by Smith et al (1938) for the estimation of selenium in urine.

2. PRECIPITATION AND COPRECIPITATION

At pH 8.0, ferric hydroxide when added to a mixture containing H_2SeO_3, will coprecipitate the selenium compound. This procedure is best used to concentrate a small amount of selenium from large amounts of material. Hirano et al (1952, 1954) used this method to extract selenium from copper alloys.

3. ION-EXCHANGE METHODS

Several groups of workers have reported the use of ion-exchange procedures to purify selenium and its compounds. Among them Yoshino (1950, 1951) used a cation-exchange step in the accumulation of selenite or selenate from sea water. Similarly, Martin and Cummings (1966) used an ion-exchange technique to separate methionine (a sulphur-containing aminoacid) and selenomethionine. This work was further developed by Martin and Gerlach (1968) to achieve the separation of selenocystine, Se-methylselenocysteine, selenomethionine and selenocystathionine.

4. SOLVENT EXTRACTION

The use of organic solvents in the purification of selenium often involves the removal of contaminants by dissolving them in the solvents. Thus, iron, copper and several other heavy metals can be separated from selenium by extracting with chilled chloroform and 6% aqueous cuproferron solution. Jordanov and Futekov (1968) recommend the use of acetophenone and 1-2M H_2SO4 for the extraction of selenium into chloroform.

5. REDUCING AGENTS

Several reducing agents have been employed in the detection of selenium, with varying measures of usefulness and adaptability. Taimni et al (1951 - 56) have suggested the use of hydrogen sulphide (H_2S) in a technique involving precipitation with removal of copper and the arsenic group metals. The conditions employed are critical however, and difficult to achieve.

Sulphur dioxide, on the other hand, is relatively easy to use (Goto et al 1952, 1953) but is not suitable for microgram quantities of selenium. The sample to be analysed is first treated with a mixture of perchloric and nitric acids, and then the nitric acid is removed by boiling. The digest is cooled and diluted with concentrated hydrochloric acid. Addition of sulphur dioxide precipitates the selenium. The precipitate is then filtered, washed and weighed. In addition some workers (De Salas, 1948) have used hydroxylamine to ensure complete reduction of any selenious or selenic acid that may be present.

Stannous chloride is a more efficient reducing agent than sulphur dioxide and is recommended (Alicino and Kowald, 1973) for the analysis of selenium concentrations of less than 1 mg/100 ml of solution. After a digestion process similar to that used in sulphur dioxide reductions, 5g of solid $SnCl_2$ is added and the mixture is heated for about fifteen minutes. Upon slow cooling of the solution, a precipitate is deposited which can be filtered through a sintered glass crucible, washed and weighed. Wiberly et al (1953) have employed this method in the separation of selenium and have found that 5 ml of a 0.6M $SnCl_2$ in 3M HCl solution is sufficient for samples containing 2-10 mg of selenium.

C. DETECTION AND IDENTIFICATION OF SELENIUM AND ITS OXIDES

1. RAPID TESTS

There are a number of quick tests that can be used to detect selenium

and to distinguish between selenite (SeIV) and selenate (SeVI) (Treadwell & Hall, 1916).

(a) Hydrogen Sulphide

When hydrogen sulphide is bubbled through a solution of selenious acid (or sodium selenite) in water or dilute acid, a lemon-yellow precipitate is obtained: $H_2SeO_3 + 2H_2S \longrightarrow 3H_2O + Se + 2S$ (or SeS_2).

The precipitate is soluble in ammonium sulphide.

If the same treatment is applied to selenic acid or sodium selenate, no reaction occurs unless the mixture is boiled with hydrochloric acid, whereupon reduction to selenite occurs, and the reaction proceeds as described above.

(b) Barium Chloride

A white precipitate of barium selenite is obtained when barium chloride is added to a neutral solution of sodium selenite.

$$BaCl_2 + Na_2SeO_3 \longrightarrow BaSeO_3 + 2\ NaCl$$

The precipitate is soluble in dilute acids.

Addition of barium chloride to sodium selenate solutions also produces a white precipitate (barium-selenate, $BaSeO_4$) which is not soluble in water and dilute acids. When boiled with concentrated hydrochloric acid, however, the solid will dissolve, with liberation of chlorine.

$$BaSeO_4 + 4HCl \xrightarrow{\triangle} BaCl_2 + H_2SeO_3 + Cl_2\uparrow + H_2O$$

This method can be used to detect selenic acid quantitatively, if the amount of chlorine given off is measured; this would give a direct indication of the quantity of barium selenate present.

(c) Copper (II) Sulphate

The reaction between selenite solutions and copper sulphate produces a greenish-blue, crystalline precipitate which is insoluble in water. Aqueous solutions of selenate, however, produce no such precipitate.

(d) Dry Test

If a selenium compound is heated at the end of a thread of asbestos in the upper reducing flame of a Bunsen burner, it will be reduced to selenium (Treadwell and Hall, 1916). If a test-tube filled with water is held above the flame, a red coating of selenium will be deposited on the glass.

This test can be extended to differentiate between compounds of selenium and tellurium as they are often confused. If a few drops of concentrated sulphuric acid are placed in a larger test-tube (large enough to hold the first tube) and the test-tube on which the selenium is deposited is placed within the larger tube, the selenium will dissolve in the acid, forming a green solution; addition of water, however, will reprecipitate elemental selenium, the red colour of which serves to distinguish it from tellurium.

$$\underset{\text{green}}{SeSO_3} + H_2O \rightleftharpoons Se\downarrow_{\text{red}} + H_2SO_4$$

(e) Concentrated Sulphuric Acid

An intensely green solution is produced when elemental selenium is heated strongly with concentrated sulphuric acid.

$$Se + H_2SO_4 \longrightarrow \underset{\text{green}}{SeSO_3} + H_2O$$

Red selenium is precipitated upon the addition of water:

$SeSO_3 + H_2O \longrightarrow \underset{\text{red}}{Se} + H_2SO_4$ (Barr et al 1968). The compound to be tested is heated with conc. H_2SO_4 in a dry test-tube that has previously been heated for 15 minutes at 170^o - 180^o (in an oil bath). A green colour indicates the presence of selenium. This test can also be used to detect selenium contamination of acids. A few drops of the acid to be tested are added to a freshly prepared solution of sulphuric acid containing a little codeine. If selenium is present, a green colouration will be apparent.

(f) Methylene Blue Test

The ability of selenium to catalyze the reduction of methylene blue by sulphide ions was first recognised by Feigl and West (1947), who using Na_2S showed that elemental selenium immediately decolorises the dye, while other elements e.g. tellurium take 15–20 minutes. The reaction is of historical interest in that it was used by Schwarz and Foltz (1957) to identify selenium as an essential trace element. Subsequently, qualitative and quantitative methods of analysis for selenium have been developed on the basis of this reaction (West and Ramakrishna, 1968); Goto et al (1952) also studied this reaction and have shown that concentration and temperature determine the limits of interference from other elements. Rhead and Schrauzer (1974) in a detailed investigation, studied this reaction in order to establish whether it may explain some of the functions of selenium in vivo. They studied the reduction of methylene blue with thiols as a model to explore the mechanism of reactions in which selenium promotes oxidation-reduction processes.

2. CHROMATOGRAPHIC PROCESSES

Although colour tests have been used to distinguish between selenium and tellurium, such as that of Burstall et al (1950) in which selenium produces an orange colour and tellurium a black colour following reduction with stannous chloride, chromatography has also been employed to detect and estimate relatively small amounts of selenium e.g. 5–45% Se in silicates. Weatherley (1956) described a process in which thiourea solution was used to detect the two elements: an orange colour was obtained with selenium and a yellow colour with tellurium; 40% hydrofluoric acid was used to develop and separate the colours on a chromatographic plate.

Another detection system using stannous chloride ($SnCl_2$) was devised by Bighi and Mantovani (1956); the solvents used were pyridine, n-propanol and water and the authors were able to distinguish H_2SeO_3

H_2SeO_4, $H_2SeS_2O_6$ and $Na_2SeS_4O_6$ by their Rf values.

3. TITRIMETRIC METHODS

Of these, the iodometric methods of which there are three, have been found to be sensitive and accurate when organic compounds are tested. Some inorganic ions (Cu, Fe) interfere by oxidising the iodide ion (Alicino and Kowald, 1973) and strong oxidising agents, if present, will also interfere. Most of these can, however, be removed.

A. IODOMETRIC METHODS

(a) Hydrochloric Acid Reduction

With a moderate excess of potassium iodide, iodine is liberated from a solution of H_2SeO_3 in hydrochloric acid:

$$H_2SeO_3 + 4HCl + 4KI \longrightarrow Se + 2I_2 + 4KCl + 3H_2O$$

The liberated iodine is ti trated with thiosulphate solution with an equivalence of Se $\rightleftharpoons$ 4I. Low concentrations of selenium are preferred so that the red deposit does not obscure the end-point of the ti tration (Alicino and Kowald, 1973).

(b) Liberation of iodine from potassium iodide

Another iodometric method for selenium estimation depends on the liberation of chlorine when hydrochloric acid is added to a solution of selenic acid.

$$H_2SeO_4 + 2HCl \longrightarrow H_2SeO_3 + Cl_2 + H_2O$$

The chlorine obtained is distilled into a dilute solution of potassium iodide at room temperature.

$$2KI + Cl_2 \longrightarrow I_2 + 2KCl$$

The equivalence obtained by this method is half of that by hydrochloric acid reduction.

Se $\rightleftharpoons$ 2I (Alicino and Kowald, 1973)

(c) Excess Thiosulphate

Selenious acid can be reduced by an excess of sodium thiosulphate solution (Norris and Fay, 1898).

$$H_2SeO_3 + 4Na_2S_2O_3 + 4HCl \longrightarrow Na_2SeS_4O_6 + Na_2S_4O_6 + 4NaCl + 3H_2O$$

The remaining sodium thiosulphate is then back-titrated with standard iodine solution.

$$2Na_2S_2O_3 + I_2 \longrightarrow Na_2S_4O_6 + 2NaI$$

The determination is carried out at room temperature and the end-point is easily visible as excess iodine will turn the mixture brown.

B. POTASSIUM PERMANGANATE TITRATION

This method is not as sensitive as the iodometric titration (Alicino and Kowald, 1973) but it can be used in the presence of moderate amounts of copper and iron; nitric and halogen acids do interfere, however, and must be removed before selenium can be measured.

$$2KMnO_4 + 5H_2SeO_3 + 3H_2SO_4 \longrightarrow 5H_2SeO_4 + 2MnSO_4 + 3H_2O + K_2SO_4$$

The excess standard permanganate solution is back-titrated with ferrous ammonium sulphate until the purple colour disappears. A large excess of phosphate ion is required to complex the Mn^{2+} and prevent its reaction with excess MnO_4^- (Barabas and Cooper, 1956).

4. SELENIUM BIOASSAY

Giasuddin (1976) devised a rat bioassay method for the estimation of selenium in foetal calf serum (FCS) which formed the basis for a medium in which cells were cultured. Selenium had been shown to have an effect on the growth of those cells grown in a low FCS media (2.5%) while no effect was observed when selenium was added to media containing 7.5% FCS. These results indicated that the FCS contained selenium and Giasuddin estimated the serum selenium by a rat bioassay method.

Weanling rats were given a basal Torula yeast diet (see Chapter 11) which was deficient in selenium and vitamin E. The rats were divided into five groups: those in one group were given 0.1 ppm selenium as sodium selenite, in their drinking water, while those in the other groups were given different doses of the foetal calf serum. Survival of the rats was used as the criterion of the protective effect of FCS against liver necrosis. 83% of the rats given the Torula yeast diet only died within forty days of the start of the experiment; those rats supplemented with 0.1 ppm Se all survived. Among the other groups, it was found that neither 0.25 ml nor 0.5 ml FCS/day/rat was sufficient to prevent high mortality rates: 83% and 67% respectively. Administration of 2.5 ml FCS per day was, however, sufficient to lower the proportion of deaths to 16%. The selenium content of FCS was calculated on the basis that 1.0 ml gives complete protection against liver necrosis and that 0.02 ppm Se in the diet also affords the same protection, a fact that had been established by Giasuddin in parallel experiments. An average daily intake of 10g diet per rat leads to a selenium intake of 0.2 μg per day per rat. 1 ml FCS could thus be said to contain 0.2 μg Se.

5. Neutron Activation Analysis

Analysis by neutron activation has been a useful and highly sensitive method for determining trace elements. The technique used involves exposure of a small area (ca. 1 cm^2 wet tissue) of wet-ashed and dried or otherwise prepared tissue to thermal neutrons or a mixture of thermal and slow neutrons (Tobias et al, 1952). Several of the elements present in the sample, including selenium, may become radioactive and the newly formed radioactive isotope may be measured by standard radiochemical techniques, which allows its decay to be followed.

Much of the literature on neutron activation analysis of selenium has been concerned with either the determination of trace impurities in high-purity selenium (Ballaux et al, 1967, 1968, 1968a, 1969)

TABLE X - 1

Radionuclides produced by a thermal neutron flux of 10^{12} neutrons/cm^2/sec on selenium (Bowen and Cawse, 1963)

Radionuclide	Specific activity of selenium after activation for one half-life (mCi/g)	Half-Life	Maximum Beta Energy (MeV)	Maximum Gamma energy (MeV)
^{75}Se	25	120 days	-	0.27 0.14 + others
^{77m}Se	97	17.5 secs	-	0.16
^{81}Se	25	18.6 min	1.60	-
All others	3	-	-	-

or the determination of trace selenium in various inorganic materials (Dams and Hoste, 1968; Conrad and Kenna, 1967) and very few papers describe in detail the estimation of selenium in biological materials by thermal neutron analysis. In their report, Bowen and Cawse (1963) stressed that only three radionuclides of selenium produced by irradiation with slow neutrons, have sufficiently high specific activity to be useful for analytical purposes (Table X-1). Many analysts prefer to use the longer-lived ^{75}Se, but its formation from the stable isotope requires a long irradiation period (half-like 120 days). In practice, the periods used are much shorter, resulting in 5-10% of the specific activity shown in Table X-1.

In an assessment of the accuracy and precision of neutron activation analysis, Bowen (1967) reported the results obtained from 29 laboratories on the analytical determination of 40 elements, including selenium, in standard kale powder. Fluorescence analysis techniques (see later) were used to compare the results for selenium, and it was concluded that neutron activation analysis gave slightly higher results than did fluorimetry.

6. ATOMIC ABSORPTION SPECTROSCOPY

The use of atomic absorption spectroscopy was first suggested by Walsh (1955) when he published his now classic paper on the analytical potential of this method. Willis (1960; 1960a) described the measurement of calcium, magnesium, sodium and potassium in blood serum by atomic absorption. Since that time, the use of this method in biological chemistry has rapidly expanded. The measurement of selenium by atomic absorption was introduced by Allan (1962) who reported on the detection limits of 28 elements in the air-acetylene flame.

In conventional flame atomic absorption, a solution of the compound containing the element to be assayed is introduced into a flame; this causes the compound to dissociate, and a population of atoms is created. Many of the atoms are in the ground or unexcited state and are capable of absorbing energy at certain resonance wavelengths which are characteristic

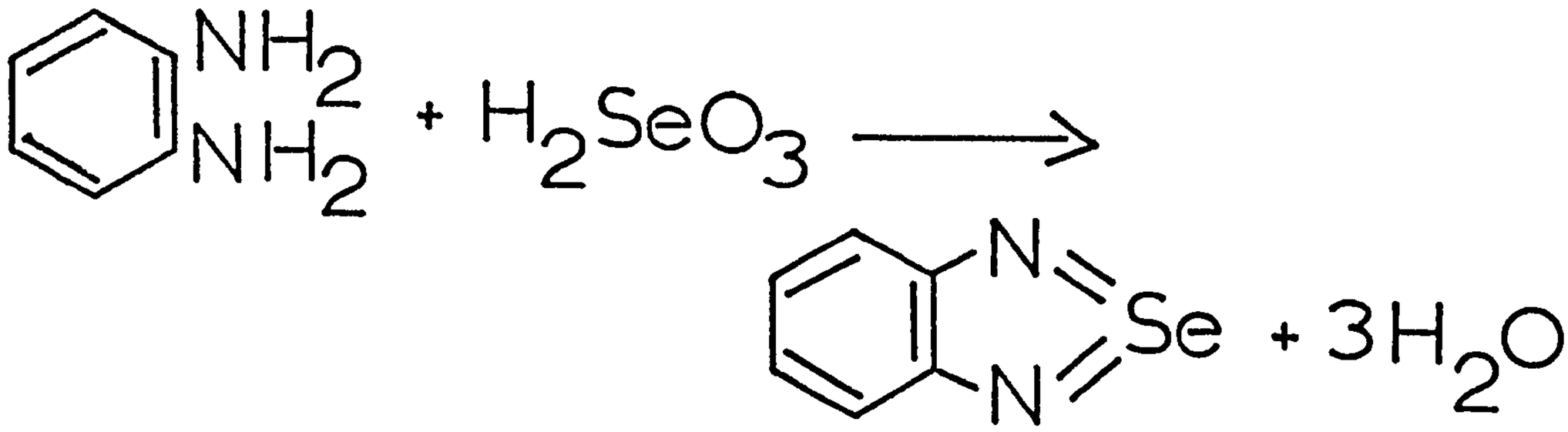
for each element. The light of these characteristic wavelengths is supplied by a hollow cathode lamp possessing a cathode composed of, or lined with, the metal under analysis. As the light passes through the atomic vapour in the flame, it is partially absorbed by atoms of the assayed metal. For analysis, a single narrow wavelength band, which is isolated by a monochromator, is used. The reduction in light energy passing through the flame caused by the atomic absorption is measured with a photo-multiplier. Thus the metal can be both identified and estimated.

There is another form of flame spectroscopy known as atomic fluorescence spectroscopy, which has been used by Winefordner and Vickers (1964) and by Winefordner and Staab (1964) for selenium analysis. The radiation from a light source is used to excite the nebulized atoms to fluorescence, and the intensity of the fluorescence serves as a measure of the concentration of selenium.

A new atomic absorption accessory recently developed by the Perkin-Elmer Corporation (1972) is a highly sensitive "selenium sampling system" which operates in conjunction with their atomic absorption spectrophotometer. Pye-Unicam (1976) have also developed a kit to work with their atomic absorption spectrophotometers which enables fast measurement of trace concentrations of arsenic and selenium. Both these adaptations depend upon the chemical reduction of the metal in the sample to their gaseous hydrides, using sodium borohydride. The hydrides are then swept to an argon hydrogen flame where the atomic absorption is measured. The Hydride Kit permits analysis and measurement of a higher proportion of the selenium present in the sample since the element is first converted to its volatile hydride. In conventional atomic absorption, using the nebuliser spray chamber, only about 12-13% of the aspirated solution passes through the flame. With the Hydride Kit, <u>all</u> the selenium in the sample passes through the flame.

7. FLUORIMETRY

A precise, accurate and comparatively rapid fluorometric method for the determination of selenium in the nanogram range has been investigated, using aromatic ortho-diamine compounds. The principle is that described by Hinsberg in 1889, who depicted the reaction between o-phenylenediamine and selenium (IV) as shown below:-



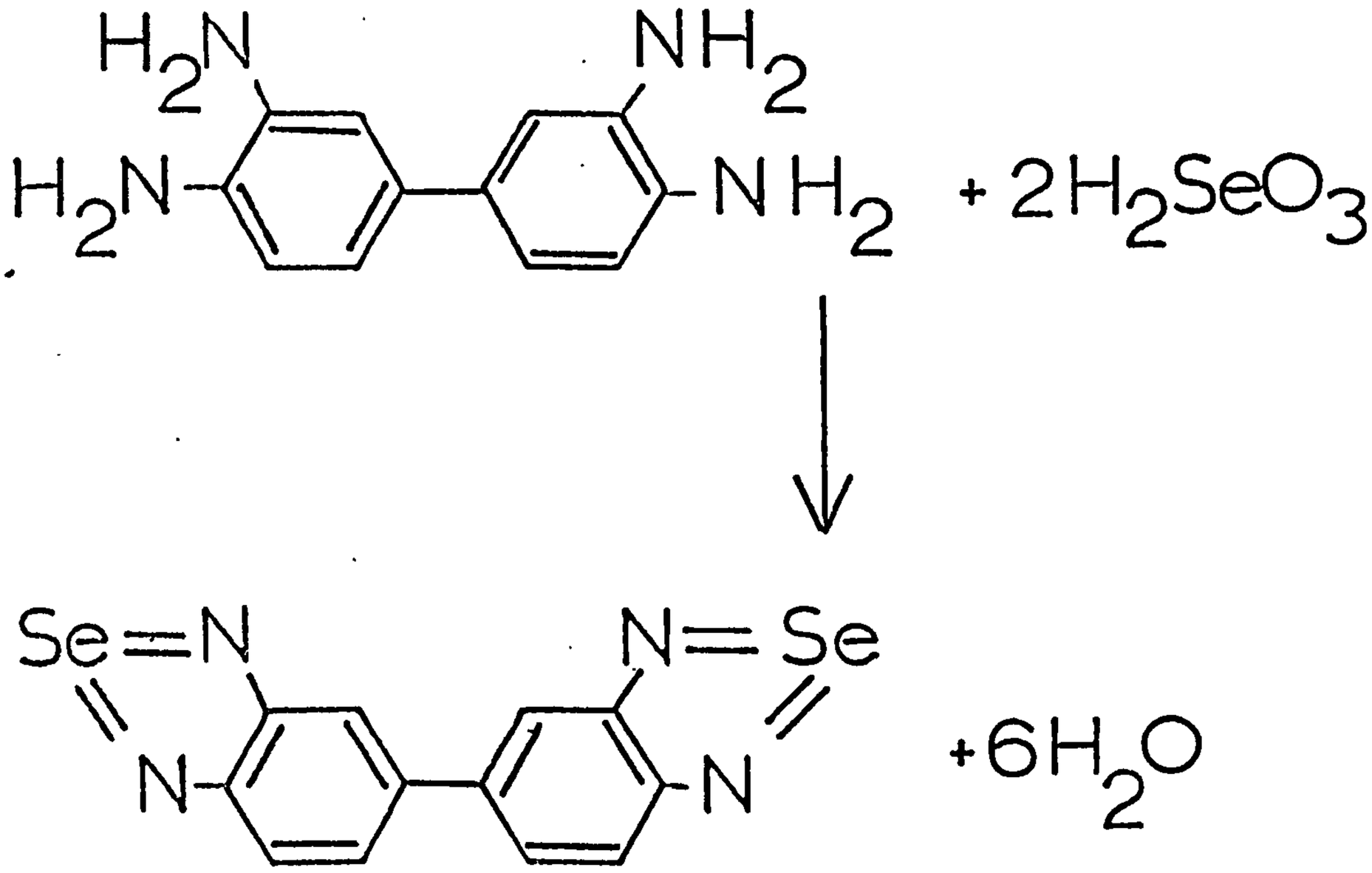
Piazselenol

The piazselenol was measured colorimetrically.

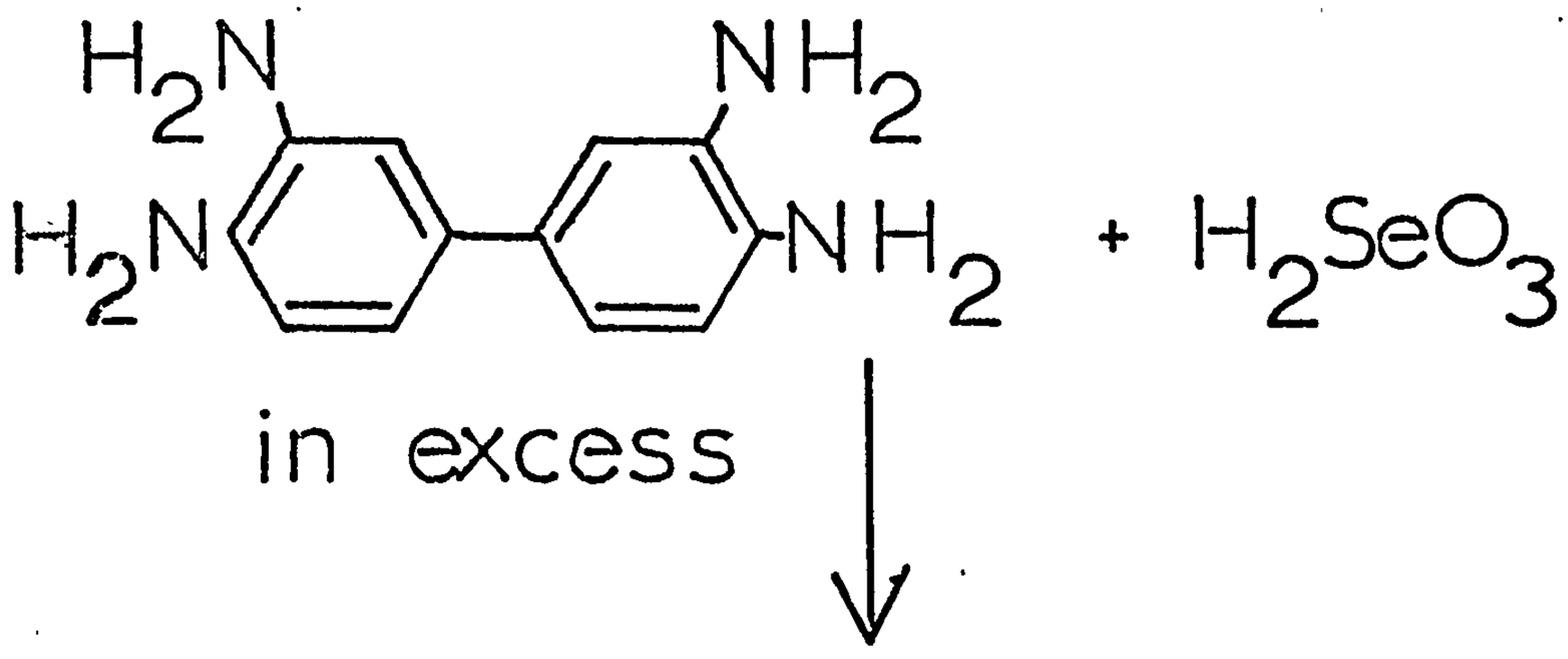
In the last fifteen years or so, a number of analytical methods for the determination of selenium have been reported that use o-phenylenediamine and its derivatives. Ariyoshi et al (1960) developed a U.V. spectrophotometric method, using o-phenylenediamine. Tanaka and Kawashima (1965) found that 4-substituted o-phenylenediamines also react with selenious acid in acid solution to form benzoselenadiazole which can be extracted into toluene. Of these reagents, 3, 4-dichloro -1, 2, phenylenediamine appears (Goto and Tōei, 1965) to be the most sensitive for the spectrophometric determination of selenium.

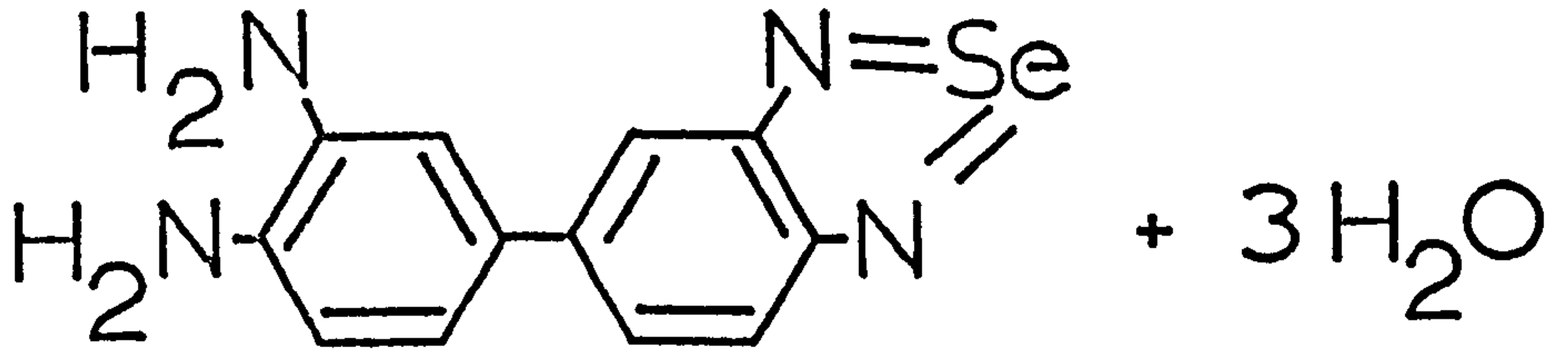
Another of the aromatic o-diamine reagents for the photometric determination of selenium is 3, 3' -diamino-benzidine (DAB). Selenium (IV) forms a yellow and very brightly coloured piazselenol

with DAB:



Hoste (1948) first described the use of this reagent in the quantitative analysis of selenium and vanadium. He described the preparation of DAB from the more easily obtainable benzidine, and its use as a qualitative drop reagent for detecting some thirty different ions. Cheng (1956) showed that the piazselenol was developed in acid solution at pH 2 to 3. Extraction by toluene at pH below 3 resulted in only partial recovery of the diphenylpiazselenol and optimum results were obtained by extracting into toluene at pH above 5. Under these conditions, the colour had an absorption maximum at 420 nm and a molar extinction coefficient of 19,900 (Cheng, 1956). Ethylenediaminetetra acetic acid (EDTA) was used to prevent interference from iron, copper and other polyvalent metals. Parker and Harvey (1961) have shown that, with excess DAB, the monopiazselenol is formed.

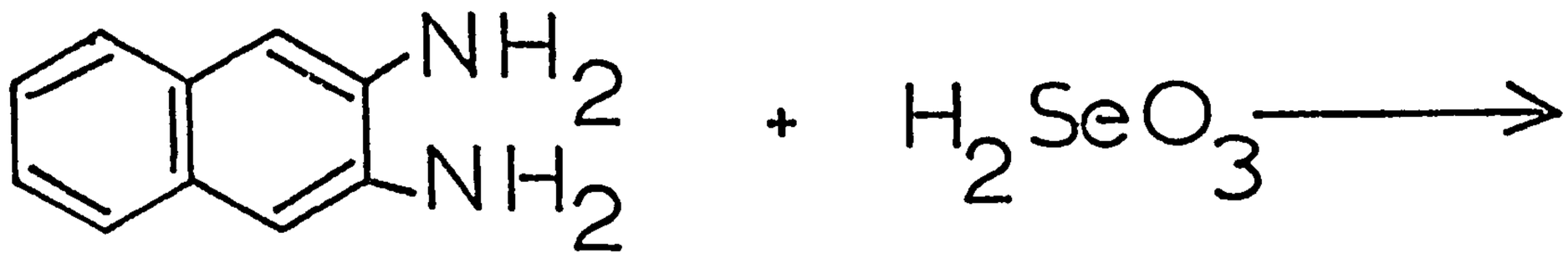




Handley and Johnson (1959), applying Cheng's method to the analysis of plant material containing added selenium, detected amounts as low as 0.25μgSe in 1.0g samples of plant material. Cummins et al (1964; 1965), in a further modification, simplified earlier DAB procedures for the determination of selenium in animal tissues. They found that the maximum colour development occurred in less than twenty minutes if the reaction mixture was heated to 60^{o} before toluene extraction. A single sample could thus be assayed in a relatively short time (15 samples in 2 hours) and assay results were comparable to those obtained by neutron activation analysis.

In addition to the use of 3, 3' diaminobenzidene as a colorimetric reagent for selenium (IV), Cousins (1960) discovered that the yellow piazselenol formed by Se with DAB is strongly fluorescent. Dye et al (1963) reported the excitation and emission wavelengths as 425 and 565 nm respectively, and Grant, in the same year (1963), developed a spectro fluorimetric assay permitting the determination of 0.01μgSe, with a standard deviation of 0.001μgSe. Watkinson (1960) has also used the fluorimetric properties of the Se-DAB complex for accurate determination of selenium and plant and animal materials by extracting the selenium from the digested sample into a mixture of ethylene chloride and carbon tetrachloride.

In 1962, Parker and Harvey introduced the reagent which is now most widely used for the fluorimetric determination of selenium - 2, 3- diaminonaphthalene (DAN). Another of the group of aromatic o-diamine reagents, DAN forms the bright red, extremely fluorescent 4, 5-benzopiazselenol.



The versatility of DAN was demonstrated by Lott et al (1963) who used it for macro-, micro- and submicro- determinations of selenium. Milligram quantities of Se were determined gravimetrically after precipitation with DAN. For microgram quantities, DAN was employed spectrophotometrically (the absorption maximum of the Se-DAN complex is at 380 nm with linear correlation of concentration and absorption up to 12μgSe); and submicrogram amounts were determined fluorimetrically.

DAN fluorimetry has been used by Watkinson (1966) and by Hoffman, Westerby & Hidiroglou (1968) to analyse the selenium content of animal and plant tissues, and also by Michael and White (1976) who worked on sulphide ores. In all cases, as little as 0.01μgSe could be detected with a standard deviation of 3-10%. Olson (1969) using a modified assay procedure, analysed plant materials for selenium and found that the results compared well with those of assays by Klein (1943), Cheng (1956) and Cummins et al (1965) and with neutron activation analysis. The method described by Olson (1969; 1975) has been recommended and accepted for adoption by the Committee on Recommendations of Referees (1969) of the

Association of Official Analytical Chemists, as an official method for the determination of selenium in plants.

8. METHOD OF SELENIUM ANALYSIS USED IN THE PRESENT WORK

The procedure used in our laboratory for the assay of selenium in rat tissues is basically that described by Olson et al (1975) and by Arthur (1977).

The samples were digested in 75 ml Quickfit stoppered boiling tubes. These tubes were chosen so that no transfer of the digests need take place during the entire process. The tissue samples (less than 1 gm weight) or fluid samples (less than 1 ml volume) were placed in the tubes with a few BDH boiling chips. 5 mls 'ARISTAR' concentrated nitric acid (BDH) were then added and the samples left for at least four hours, usually overnight.

The following day, 2.5 ml of 60% perchloric acid (BDH A.R. Food Analysis grade) was added to the digests; the tubes were attached to a Quickfit manifold (Fig. X-A) and heated on a multi-point Bunsen burner. A trap containing concentrated potassium hydroxide solution and a few drops of cresol red solution which indicated when it was necessary to change the KOH solution, was fitted on to one end of the manifold and a water pump was used to draw the toxic nitric acid fumes which were given off during heating, through the alkaline solution. There was thus no necessity to carry out the digestions in a fume cupboard; a bench setting was adequate.

Heating was continued for two to three hours during which time all the nitric acid should have boiled off and dense white fumes of perchloric acid appeared. The mixture was then allowed to reflux for thirty minutes or so. This method has an advantage over several others used in selenium estimation in that inorganic selenium is not appreciably volatilized during digestion in a mixture of nitric and perchloric acids

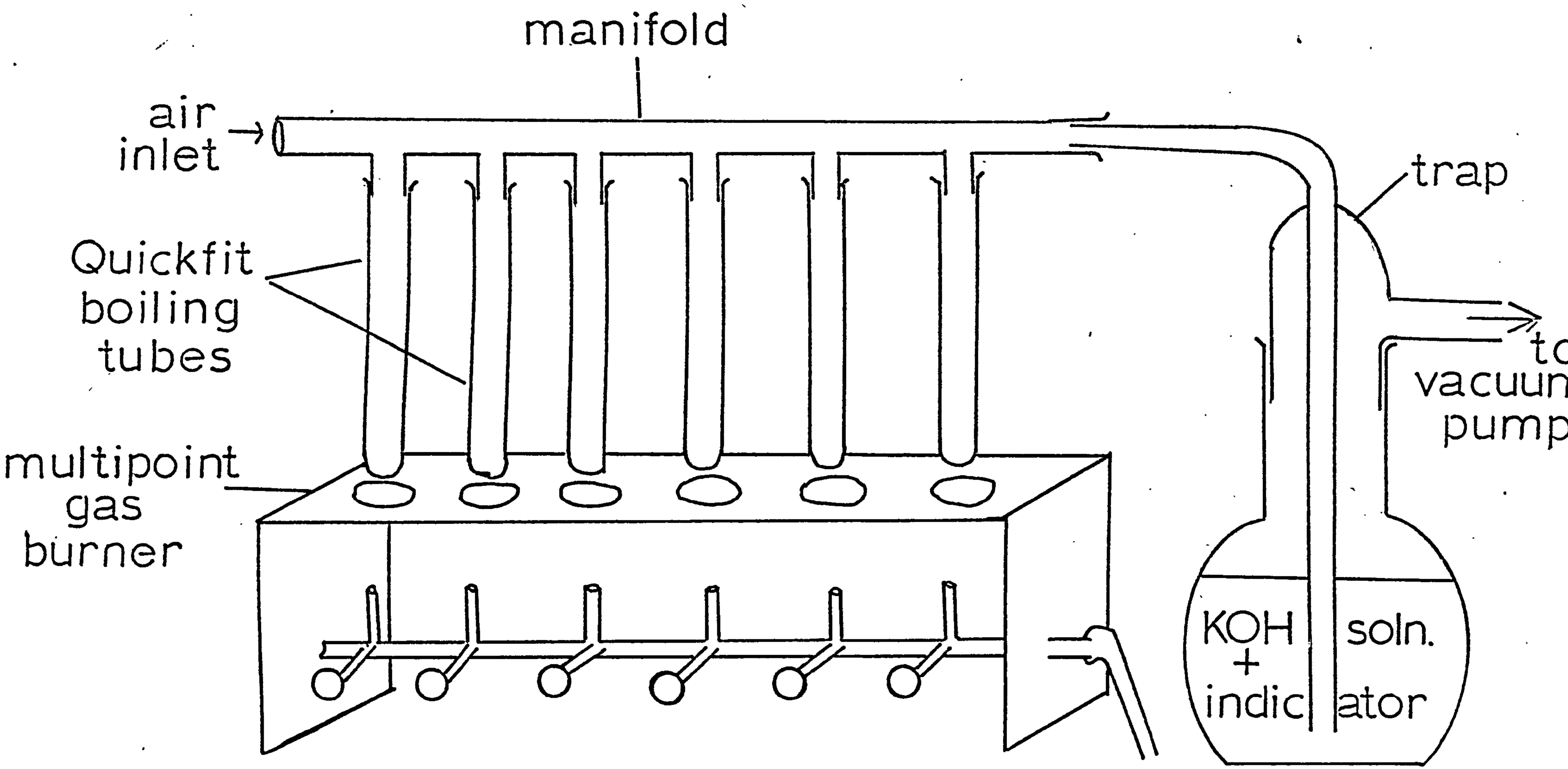


Fig. 10-A. Apparatus used in fluorometric determination of selenium.

(Bowen and Cawse, 1963; Gorusch, 1959). In the presence of a large excess of organic matter, however, charring occurs (this is characteristic of non-oxidizing or reducing solutions) and some protein-associated selenium compounds might be hydrolyzed to volatile selenides (Peterson and Butler, 1962). Gorusch (1959) and Grant (1963) have shown that under such conditions, significant amounts of selenium could be lost through volatilization, possibly by the formation of hydrogen selenide. It was, therefore, necessary to maintain strongly oxidizing conditions throughout the digestion.

Grant (1963) also showed that boiling perchloric acid slowly oxidises selenite to selenate. Therefore, after the digests had been heated, 2.5 ml of 10% conc. hydrochloric acid was very carefully added to the digests: this minimised spitting and possible loss of contents from the tube, as the digests were still hot. The hydrochloric acid drove off excess nitric acid and ensured that any of the selenium present as selenate was converted to selenite. 5 ml of a solution of 0.024 M EDTA and 0.36 M hydroxylamine hydrochloride (9g EDTA-Na_2 .$2H_2O$ and 25g Ho. NH_3 Cl in 1 litre deionised water) was then added to each digest to prevent interference from other metals. This was followed by the addition of three drops of cresol red indicator which has two colour changes: from red to yellow in the pH range 0.2 to 1.8 and from yellow to violet-red in the range 7.2 to 8.8; it thus has a red colour on immediate addition to the digests. 40% conc. ammonia solution was then added until the indicator turned yellow and 10% conc. HCl was added until the indicator just started to turn pink/orange. This brought the digests to pH 1.5 - 2.5 which was optional for the formation of the 2, 3- diaminonaphthalene - selenium complex. The digests were then diluted to 50 ml.

5 mls of a 0.003M DAN solution (500 mg of 2, 3 - diaminonaphthalene dissolved in 1 litre 0.1M HCl) was added to each digest and the tubes were

stoppered and shaken. They were then heated in a covered water bath at 50° for thirty minutes. This enabled full development of the DAN-Se complex without exposure of the light sensitive DAN to sunlight.

After thirty minutes, the tubes were cooled by plunging them into cold water for five minutes. 6ml cyclohexane (BDH 'Special for fluorimetry'; or twice-distilled Lab-Grade cyclohexane) was added to each digest and the tubes were shaken for 20 seconds to extract the DAN-Se complex. The mixture was then allowed to separate by standing for fifteen minutes and the cyclohexane layer was removed into a clean test-tube using a pipette.

The fluorescence of the complex was then measured and compared to that obtained using reference solutions. These standards were prepared in deionised water so that 0.1 ml solution contained the required amount of selenium. As they contained no organic matter, there was no need to digest them. 2.5 ml of 60% perchloric acid was added to them, followed by 5 ml of hydroxylamine - EDTA solution. They were then treated in exactly the same manner as the digested samples.

A. EXPERIMENTAL POINTS

All glassware used was soaked in a 50:50 mixture of conc. nitric acid: deionised water for at least thirty minutes. They were then rinsed twice in freshly distilled water and then in double-distilled water which had been deionised.

All the chemicals used were A.R. grade obtained from BDH chemicals with the following exceptions:-

(a) Nitric Acid: 'ARISTAR' grade with a maximum content of 5 ppm of non-volatile matter, as opposed to 10 ppm in 'ANALAR' grade.

(b) Cyclohexane: 'Special for fluorimetry' grade with potentially fluorescent substances removed. Also, in the later experiments described in this thesis, double-distilled Laboratory grade cyclohexane was used and found to give acceptable blanks. This had the merit of being considerably cheaper than the 'Special' cyclohexane.

(c) 2, 3 Diaminonaphthalene: obtained from the Aldrich Chemical Company, with a minimum assay of 98%.

B. PREPARATION OF REFERENCE SOLUTION

Standard solutions of selenious acid in double-distilled, deionised water were required such that 0.1 ml (the volume used for all the solutions) contained between 0.01 and 0.6 μgSe. In order to obtain reproducible results based on accurate weighing, an initial solution was prepared which was further diluted in order to give the reference solutions.

Acid-washed 10 ml volumetric flasks were used for storage of the standard solutions. Aliquots of the initial solution were then measured into the flasks and made up to the 10 ml mark, thus yielding solutions with known selenium contents in the 0.01 - 0.6 μg/0.1 ml range.

CALCULATIONS

The initial solution contained 0.0164 g H_2SeO_3 in 1 litre deionised water.

6 mls of this solution would, for example, contain:

$$\frac{0.0164}{1000} \times 6 \text{ mg} = 0.0984 \text{ mg } H_2SeO_3 \qquad \left\{ \begin{array}{ll} \text{m w} & H_2SeO_3 \quad 128.97 \\ \text{a w} & Se \quad\quad 78.96 \end{array} \right\}$$

$$0.0984 \text{ mg } H_2SeO_3 \equiv \frac{0.0984 \times 78.96}{128.97} \text{ mgSe}$$

$$= 0.0602 \text{ mgSe in 6 mls initial solution}$$

If 6 mls were diluted in a 10 ml volumetric flask, the concentration would become:

0.0602 mgSe in 10 mls solution

= 0.00602 mgSe/ml soln

= 0.000602 mgSe / 0.1 ml soln = 0.6 μgSe

Further dilutions of the initial solution were as shown in Table X-II.

TABLE X-II

Dilution of initial solution into 10 ml volumetric flasks to yield standard selenium solutions.
Initial solution ≡ 0.0164g H_2SeO_3 per litre

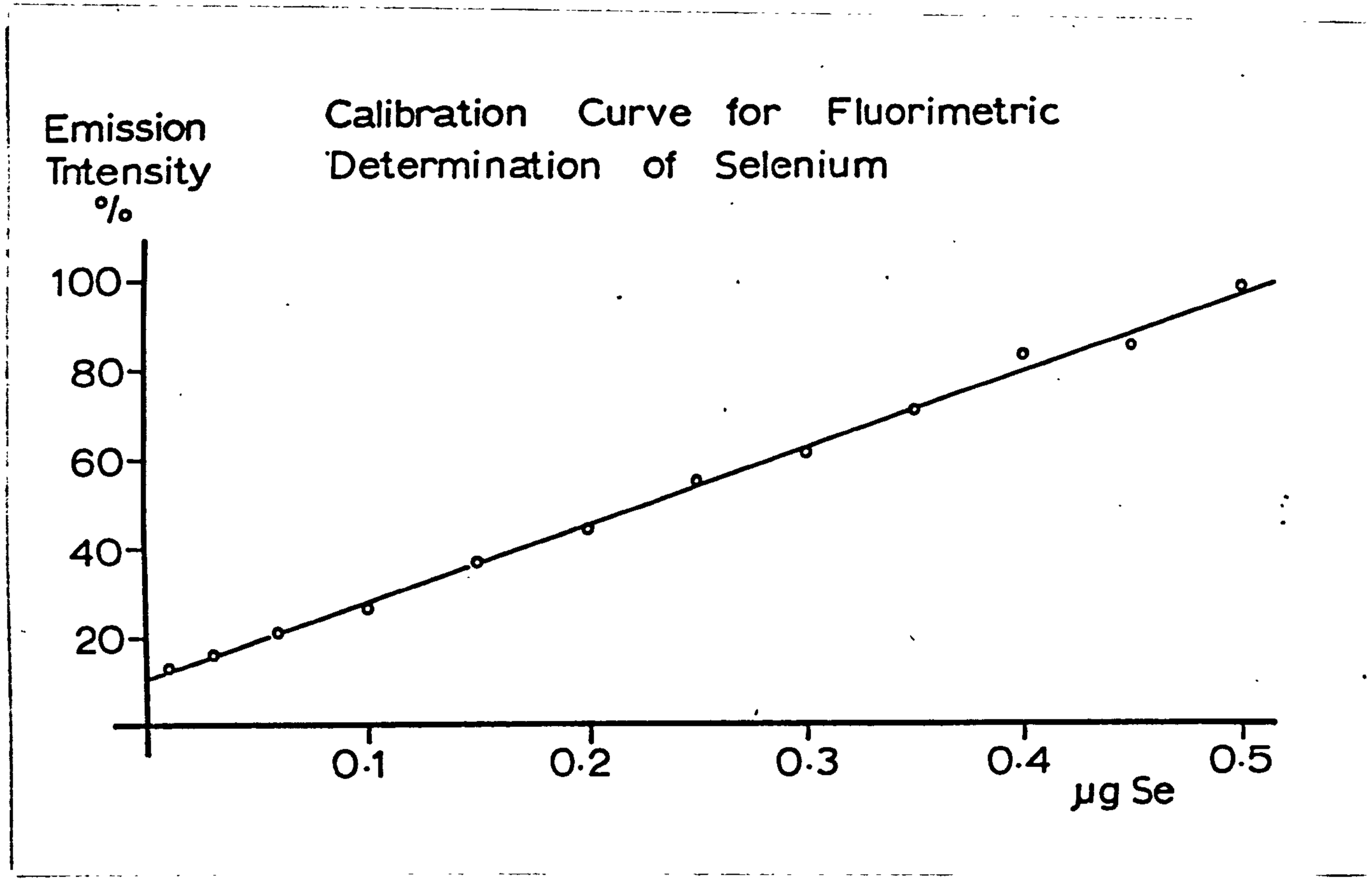
Volume of initial solution diluted to 10 mls	Concentration of H_2SeO_3 in 10 mls standard solution	Concentration of H_2SeO_3 in 0.1 ml of standard solution	Concentration of Se in 0.1 ml standard solution
0.1 ml	0.0016 mg	0.0163 μg	0.01 μg
0.3 ml	0.0048	0.0489	0.03
0.6 ml	0.0098	0.0978	0.06
1.0 ml	0.0163	0.1633	0.10
1.5 ml	0.0245	0.2445	0.15
2.0 ml	0.0326	0.3260	0.20
3.0 ml	0.0490	0.4900	0.30
4.0 ml	0.0653	0.6533	0.40
5.0 ml	0.0817	0.8167	0.50
6.0 ml	0.0978	0.9780	0.60

The fluorescence of the red piazselenol formed by selenium and DAN is linear with selenium concentration over the concentration range 0.1 to 6 μgSe per ml solution as shown in Fig. X-I. The absorption and emission spectra of the complex were obtained using a Perkin-Elmer Fluorescence Spectrophotometer, model 204A. With the emission wavelength set at 525 nm and the excitation wavelength dial set to scan, the

Fig. X-I.

Calibration curve for the fluorescence of the DAN-Se complex using standard solutions of selenious acid.

Excitation wavelength 365nm. Emission wavelength set to 515 nm.



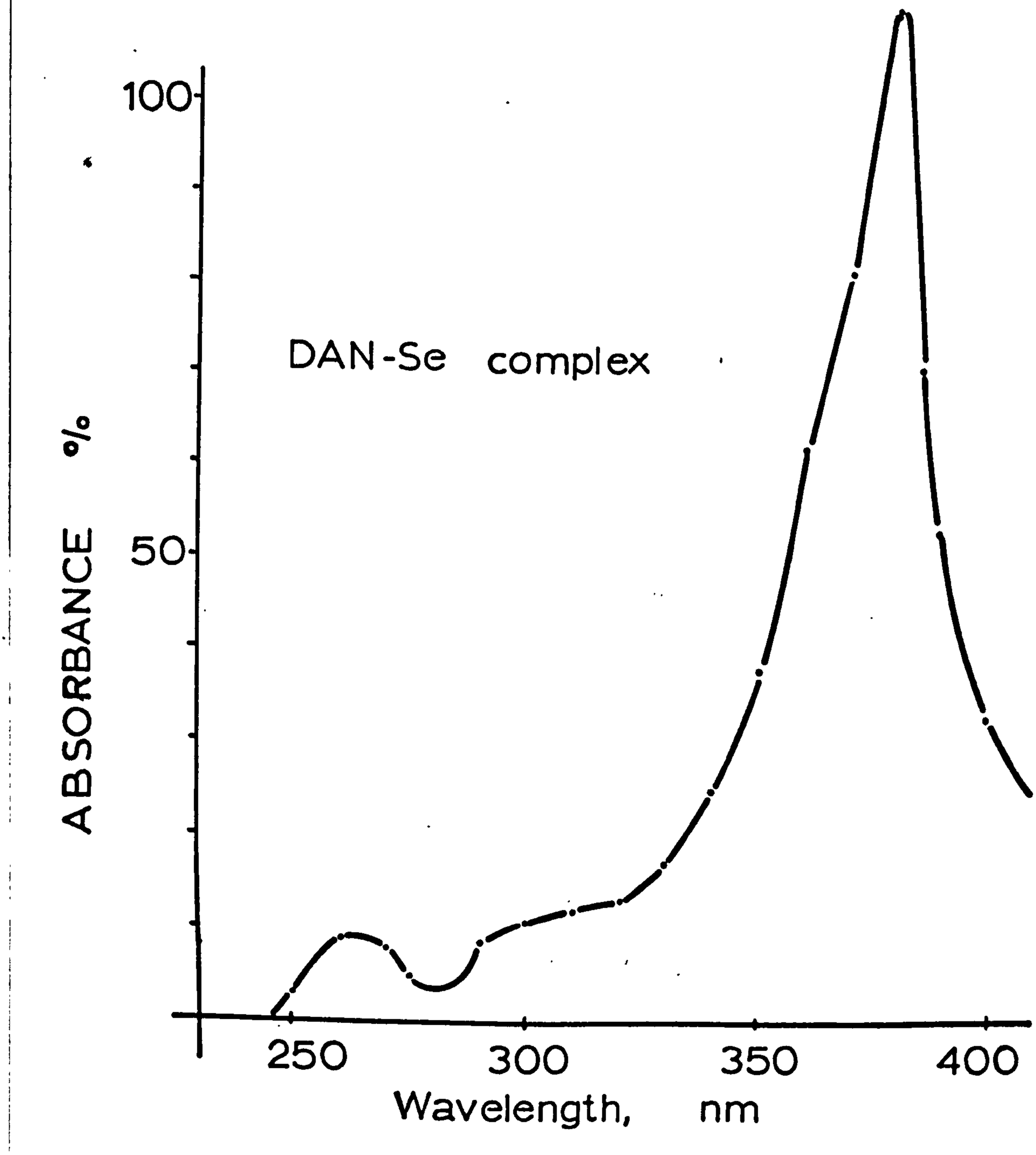


Fig. X-II. ,Absorption spectrum of the DAN-Se complex.

A solution containing 0.5 µg Se/ml was used ; the complex was extracted into cyclohaxane and was analysed in a fluorescence spectrophotometer.

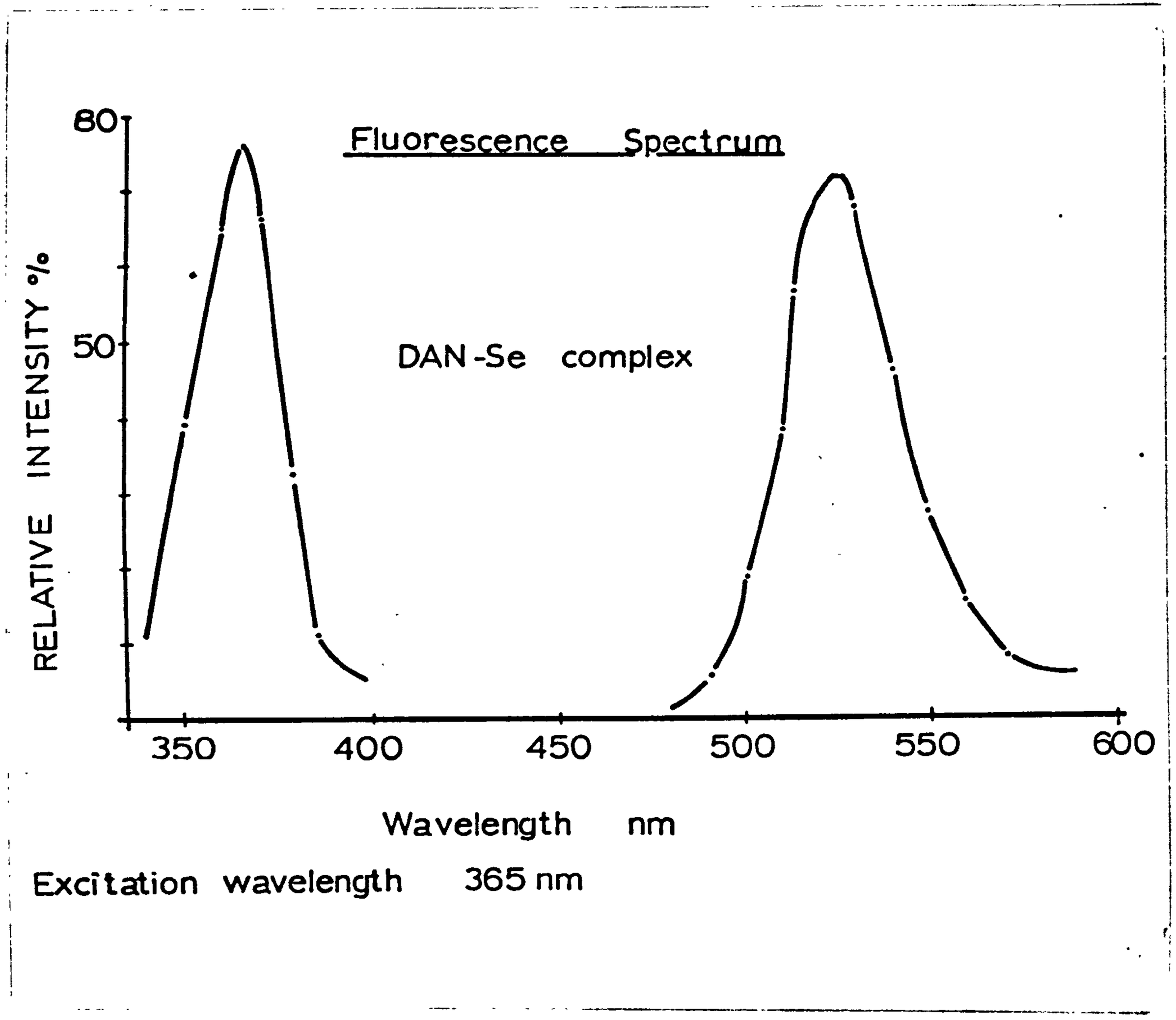
Emission wavelength 515 nm. Excitation wavelength set to scan.

Fig. X-III.

Fluorescence spectrum of the DAN-Se complex.

The complex obtained from 0.5μg Se was extracted into cyclohexane and analysed in a fluorimeter.

Excitation wavelength 365 nm. Emission wavelength set to scan.



absorption spectrum (Fig. X-II) was recorded. It shows an absorption maximum in the range 365-380 nm.

Then, with the excitation wavelength set at 365 nm, the fluorescence spectrum of the complex was recorded by scanning the emission spectrum (Fig X-III). There was an emission band at 515-530 nm with a peak at around 525 nm.

C. RECOVERY EXPERIMENTS

Two experiments were carried out to determine the percentage of a known quantity of selenium which could be detected by this method, and the effect of concentration on recovery limits.

Experiment 1

A solution containing 0.0020 g H_2SeO_3 in 20 mls was used as the stock solution. Two other solutions were prepared by diluting the stock solution with deionised water. These were treated as the test solutions:

0.0020 g H_2SeO_3 ≡ 1.224 mgSe in 20 mls

= 61.2235 μgSe/ml

$\frac{1}{30}$ dilution ≡ 2.0408 μgSe/ml or 0.2041 μgSe/0.1 ml

$\frac{1}{15}$ dilution ≡ 4.0816 μgSe/ml or 0.4082 μgSe/0.1 ml

Each test solution was analysed in duplicate by the DAN-Se fluorescence method. Standard selenium solutions were used to calibrate the emission readings on the fluorimeter and the selenium content of the test solutions were estimated from the calibration curve:

Average of results:	Concentration of Se Detected by Fluorimetry	% of calculated Se-content
$\frac{1}{30}$ dilution	0.1949 μgSe/0.1 ml	95.54%
$\frac{1}{15}$ dilution	0.3973 μgSe/0.1 ml	97.32%

Estimation of selenium by DAN-Se complex fluorescence thus gives results to within 5% of the calculated value.

Another experiment was carried out to determine the recovery after the selenium solution had undergone the nitric and perchloric acid digestion process.

Experiment 2

The 1 in 15 dilution of the stock solution prepared for the above experiment was used in this experiment also. Six 0.1 ml samples of the diluted solution were added to the boiling tubes used in the digestion process. The test solutions were treated in the same manner as digests i.e. nitric acid was added and they were left to stand overnight. They were heated with perchloric acid the next day and then treated as described above in the 'Method' section.

Results:

0.1 ml of a $\frac{1}{15}$ dilution of the stock solution contained 0.4082μgSe as calculated.

Comparison with standard selenium solutions gave the following results:-

	Selenium detected (μg)	Selenium Calculated (μg)	% of calculated content
1)	0.3979	0.4082	97.86%
2)	0.4106	0.4082	100.60%
3)	0.3876	0.4082	94.95%
4)	0.3957	0.4082	96.95%
5)	0.4069	0.4082	99.69%
6)	0.3905	0.4082	95.67%

These results confirm those of Bowen and Cawse (1963) since they demonstrate that digestion in a mixture of nitric and perchloric acids does not appreciably increase in the percentage of selenium volatilised

from a sample under investigation.

D. SPECIFICITY OF THIS ASSAY METHOD FOR Se(IV)

The aromatic o-diamine reagents used for the detection and estimation of selenium have been described as specific for selenium (IV) compounds. Hoste and Gillis (1955) first noted this when they observed that the yellow colour reaction of 3, 3'-diaminobenzidine (DAB) only occurred when selenium (IV) compounds were used. Subsequently, other workers using 2, 3-diaminonaphthalene (DAN) only employed selenious acid (H_2SeO_3) or its salts in the preparation of standards for selenium estimation.

The following experiment was designed to investigate the possibility that DAN could be used to detect and estimate forms of selenium other than Se(IV).

Experiment 3

This experiment was divided into two parts: the first part deals with the possible detection of selenium in sodium selenate (Na_2SeO_4), sodium selenide (Na_2Se) and DL-selenocystine. Sodium selenite was used as a marker. The second part of the experiment concerns the preparation and investigation of silver selenide Ag_2Se (q.v. Chapters 13-16).

Detection of sodium selenate, sodium selenite, sodium selenide and DL-selenocystine.

Standard solutions of these compounds in deionised water were prepared, so that the solution under test contained between 0.1 and 0.35 μgSe in 0.1 ml solution. These being such small quantities, the same method used in the preparation of the reference solutions was used here viz. the preparation of an initial or stock solution and further dilution of this to the test solutions. In this experiment a 1 in 100 dilution (0.1 $\longrightarrow$ 10 ml) of the stock solution to test solution was made. Reference solutions containing 0.01 to 0.6 μgSe (as H_2SeO_3) per

0.1 ml solution were used to calibrate the fluorescence spectrophotometer.

Each compound was tested in triplicate.

Results

Se compound under investigation		Calculated Se content of 0.1 ml solution(μg)	μgSe detected by DAN assay	% of calculated value	Average
Sodium Selenate Na_2SeO_4	1.	0.3099 μg	0.0030	0.968%	0.968%
	2.	0.3099	0.0042	1.355	
	3.	0.3099	0.0018	0.581	
Sodium Selenide Na_2Se	1.	0.2156	0.1131	52.458	53.107
	2.	0.2156	0.1311	52.458	
	3.	0.2156	0.1173	54.406	
DL-Selenocystine $C_6N_2O_4H_{12}Se_2$	1.	0.1233	0.0424	34.388	35.307
	2.	0.1233	0.0447	36.253	
	3.	0.1233	0.0435	35.279	
Sodium Selenite Na_2SeO_3	1.	0.3500	0.3386	96.743	101.924
	2.	0.3500	0.3658	104.514	
	3.	0.3500	0.3658	104.514	

The results clearly show that selenium in sodium selenate ((Se(VI)) was not detectable by DAN-Se complex fluorimetry; Less than 1% of the selenium under test was detected. On the other hand, the high percentage detection of sodium selenite (102%) showed that this fluorimetric assay was capable of quantitative results with Se(IV). In both sodium selenide and DL-selenocystine, selenium presumably exists as Se(II) and relatively high, though not quantitative, detection ratios were obtained for these compounds. It should be noted, however, that the Merck Index

(1977) lists sodium selenide as a compound that "decomposes in water". It is, therefore, possible that this "decomposition" is in fact a reaction which alters the oxidation state of the element. In any case, it was obvious that this assay primarily detected Se(IV) and was not at all effective when other oxidation states of selenium were presented.

Preparation and Estimation of Silver Selenide

Silver is a stress factor in vitamin E-deficient rats and was used in many of the experiments to be discussed later in this thesis (Chapters 13-16); it was, therefore, useful to know whether this method for the determination of selenium, could detect selenium which is complexed to silver, as silver selenide (Ag_2Se). This compound is particularly important as its formation may be the way in which silver produces symptoms of selenium deficiency *in vivo*.

The method of preparation used was that described by Diplock *et al* (1973) in their experiments on the nature of the acid-volatile selenium in rat liver. These workers reported that $H_2{}^{75}Se$ was trapped in 0.1 M $AgNO_3$ solution while $(CH_3)_2{}^{75}Se$ was trapped in 8 M HNO_3. The acid-labile selenium of a rat liver homogenate behaved in a manner similar to that of hydrogen selenide. $H_2{}^{75}Se$ was produced by treating $Na_2{}^{75}SeO_3$ with zinc dust and concentrated hydrochloric acid.

$$Na_2{}^{75}SeO_3 \longrightarrow H_2{}^{75}Se(g) \uparrow$$

$$\uparrow 2H$$

$$\uparrow Zn + HCl$$

The gas generated was trapped by Diplock *et al* (1973) in silver nitrate solution, thus producing silver selenide.

$$H_2{}^{75}Se + 2AgNO_3 \longrightarrow Ag_2{}^{75}Se + 2HNO_3$$

For the preparation of silver selenide, non-radioactive or carrier selenious acid (H_2SeO_3) with ^{75}Se-sodium selenite added as tracer was used to generate $H_2Se + H_2{}^{75}Se$. Analysis of the amount of ^{75}Se lost as

$H_2{}^{75}Se$ and that trapped as $Ag_2{}^{75}Se$ would, therefore, indicate the total quantity of silver selenide produced.

Experimental Method

0.055g H_2SeO_3 was dissolved in 1.0 ml water in a polystyrene vial (A) suitable for use in a γ- isotope counter. To this was added 1.5 ml concentrated hydrochloric acid and 0.05 ml sodium ^{75}Se-selenite of sufficient radioactivity to give an acceptable counting rate for the experiment; the radioactivity of the contents of the vial was determined. 3.0 ml of 0.1 M silver nitrate solution was added to a separate, clean vial (B) and the radioactivity of this tube was also determined. The tubes were connected by polythene tubing as described by Diplock et al (1973) and O_2- free N_2 was bubbled through the reaction vial A, across the connecting tubing and through the $AgNO_3$ solution in vial B. Thus, hydrogen selenide produced in vial A would be carried by the flow of nitrogen into the trapping solution in vial B. The reaction was started by the addition of about 350 mg of zinc dust to vial A and, after fifteen minutes, the tubes were removed for further γ-isotope measurement.

0.1 ml of the silver nitrate trapping solution and 0.1 ml of the contents of the collecting vial B after the reaction, were analysed using the DAN-Se fluorimetric assay described earlier. Comparison of the selenium content of vial B before and after the reaction would, therefore, indicate whether any detectable selenium had been trapped in the vial.

The experiment was carried out in duplicate and the results are given below:-

^{75}Se radioactivity counts:-

EXPERIMENTAL TUBE (1)				EXPERIMENTAL TUBE (2)			
REACTION Vial A		COLLECTION Vial B		REACTION Vial A		COLLECTION Vial B	
^{75}Se before expt. (cpm x 10^{-5})	^{75}Se after expt. (cpm x 10^{-5})	^{75}Se before expt. (cpm x 10^{-5})	^{75}Se after expt. (cpm x 10^{-5})	^{75}Se before expt. (cpm x 10^{-5})	^{75}Se after expt. (cpm x 10^{-5})	^{75}Se before expt. (cpm x 10^{-5})	^{75}Se after expt. (cpm x 10^{-5})
3.39	1.74	.00079	0.23	3.37	1.82	.00086	0.25

Calculations

Tube (1)

^{75}Se initial count (A) = 3.39×10^5 before reaction

^{75}Se residual count (A) = 1.74×10^5 after "

Lost ^{75}Se = 48.78% of initial count

Non-radioactive selenite:

Initial quantity H_2SeO_3 = 0.055g $\equiv$ 4.27×10^{-4} moles

Percentage of ^{75}Se volatilized = 48.78%

Therefore, 48.78% of non-radioactive selenite was also volatilized.

48.78% of 4.27×10^{-4} moles = 2.08×10^{-4} moles H_2SeO_3

One mole H_2SeO_3 will produce one mole H_2Se

Therefore, 2.08×10^{-4} moles H_2Se were produced

2.08×10^{-4} moles $\equiv$ 0.0168.g H_2Se $\equiv$ 0.0164g Se

cpm ^{75}Se trapped in $AgNO_3$ solution = $(0.25 - 0.00086) \times 10^5$

= 14.1% of the total ^{75}Se volatilised.

Thus, 14.1% of 0.0164g Se should be trapped in 3 mls silver nitrate solution as Ag_2Se.

14.1% x 0.0164g = 2.31 mg Se

0.1 ml should contain 77.15 μg Se

Tube (2)

A similar calculation for experimental tube (2) showed that the expected amount of selenium in 0.1 ml of the collection fluid was 78.08 μg Se

Fluorimetric analysis for selenium

The silver nitrate solutions in the collection vials B were analysed for selenium using DAN fluorimetry, both before and after passage of H_2Se through the solution. The results are shown below:

$AgNO_3$ solution

EXPERIMENT 1		EXPERIMENT 2	
Selenium detected in 0.1 ml of $AgNO_3$ solution (μg)		Selenium detected in 0.1 ml of $AgNO_3$ solution (μg)	
Before Reaction	After Reaction	Before Reaction	After Reaction
0.0000	0.0664	0.0002	0.1009
0.0000	0.0706	0.0001	0.0768
Av: 0.0000	0.0685	0.00015	0.0888

	Net increase in Se detected due to passage of H_2Se through 0.1 ml solution.	Calculated amount of selenium in 0.1 ml solution after H_2Se has been bubbled through it.	Percentage of calculated selenium which was detected by DAN fluorimetry.
1.	0.069 μg	77.147 μg	0.089%
2.	0.089 μg	78.079 μg	0.114%

These results show that selenium as silver selenide cannot be quantitatively detected by DAN-Se fluorimetry. The tracer studies described above using ^{75}Se proved that there was some selenium trapped in the silver nitrate solutions; it could not be detected, however, by the fluorimetric assay.

PART III

INDIVIDUAL MAJOR EXPERIMENTS

CHAPTER II

The influence of dietary vitamin E and selenium on the metabolism of selenium by rat liver.

Aim: The possible effects of dietary vitamin E and selenium on the methylation of ^{75}Se in rat liver were examined in an attempt to determine whether these dietary factors affected the intracellular localisation of selenium-metabolising enzymes. (Coker and Diplock, 1978)

Procedure: Twelve weanling, male Wistar rats (50 - 60g) were divided into four groups and given the vitamin E - and selenium - deficient diet based on Torula yeast (Diet T) with or without supplements as indicated below:

Group 1 : Diet T only. Distilled water for drinking. (Diet T: Table II-I.)

Group 2 : Diet T. 0.1ppmSe in drinking water. (T+Se)

Group 3 : Diet T + 100 mg α- tocopherol/kg diet (T+E)

Group 4 : Diet T + E; 0.1ppm Se in drinking water (T+E+Se)

(Note: the Se was added to the drinking water as Na_2SeO_3)

The animals were maintained on their respective dietary regimes for 20 - 26 days after which time they were killed by cervical dislocation, and their livers removed into ice-cold 0.25M sucrose + 10^{-4}M EDTA solution. 20% homogenates were prepared using the technique described by Caygill et al, (1971). The homogenizer used is a modification of that described by Aldridge, et al, (1960) in which a conical nylon pestle engages the nylon base of a glass tube. Caygill et al (1971) reported that greatest uniformity was achieved by using four passes of the homogeniser pestle. The preparation of liver fractions is described in greater detail in Chapter 8; briefly, the method used was as follows. The homogenates were centrifuged at 9000g

TABLE 11 - 1

Vitamin E - and Selenium - deficient Torula yeast diet

DIET T.

*	Torula Yeast	30.0 %
	Lard	5.0 %
	Glucose	15.0 %
	Sucrose	47.0 %
	Salt Mixture	3.02 %
**	Vitamin Mixture	0.4 %

THE SALT MIXTURE SUPPLIED:-	(g/kg)
$NaH_2PO_4 \cdot 2H_2O$	6.5
$CaCO_3$	17.5
KCl	3.5
$MgSO_4 \cdot H_2O$	2.25
Ferric Citrate	0.15
$MnSO_4 \cdot 4H_2O$	0.2
$ZnSO_4 \cdot 7H_2O$	0.06
KI	0.0003
NaF	0.00025
$(NH_4)_6 Mo_7 O_{24} \cdot 4H_2O$	0.002
$CoSO_4 \cdot 7H_2O$	0.01
$Al_2(SO_4)_3 \cdot K_2SO_4 \cdot 24H_2O$	0.0007
$CuSO_4 \cdot 5H_2O$	0.02

* Obtained from Lake States Yeast and Chemical Division of St. Regis Paper Company, Rhinelander, Wisconsin, U.S.A.

** The same vitamin mixture as for the vitamin E - deficient diet (Table 13-1) was used.

for ten minutes and the supernatant fraction (S_1) was removed and centrifuged at 100,000g for one hour using a MSE superspeed '65' Ultracentrifuge. The 100,000g supernatant (S_2) was removed and the pellet, composed mainly of microsomes, was resuspended in 0.25M sucrose solution and re-centrifuged at 100,000g for one hour. The washed pellet, called microsomal fraction (Ms) was resuspended in 0.25M sucrose solution.

Dimethyl Selenide (DMSe) Generation

The formation of dimethyl selenide from an inorganic salt was studied as an example of selenium metabolism in rat liver. Sodium selenite solution was added to an incubation mixture at 37° containing the liver fraction under test, reduced glutathione (GSH), glucose -6-phosphate, S-adenosyl-methionine and necessary enzymes and co-factors as described in Chapter 8.

The procedure given by Ganther (1966) and by Diplock et al (1973) was then followed, resulting in the formation of dimethyl selenide which was swept out of the incubation tube by a stream of nitrogen. The reaction was terminated after thirty minutes.

Radioactive selenium as $Na_2{}^{75}SeO_3$ was added to the reaction tubes in minute quantities, and used as a tracer for the larger amount of selenite present (see Chapter 8 for discussion of this procedure). Thus, dimethyl selenide generation was calculated from the loss of ^{75}Se from the reaction vial during the incubation period. Units of activity of the liver fractions were defined as 'the percentage of the total ^{75}Se in the reaction vial lost during the thirty minute incubation, expressed per g of liver'.

The liver fractions which were tested in DMSe synthesis experiments were:-

S_1	9000g supernatant fraction
Ms	Suspension of washed microsomes
S_2 + Ms	A recombination of washed microsomes and 100,000g supernatant fraction.

Rats were killed 20, 23 and 26 days after the start of the experiment and each fraction was tested in triplicate on each experimental day. The results are presented in Fig. 11-1. When vitamin E was not included in the diet, selenium metabolism was greatly enhanced by inclusion in the diet of 0.1 ppm selenium. This effect was observed in all the liver fractions tested and was established statistically ($p > 0.01$) for the S_2 + Ms combination fraction, using the Student's t test.

Table 11-2 shows the mean $\pm$ S.D. of nine determinations of the DMSe generating activity of each liver fraction. When the diet was supplemented with 100 mg/kg of vitamin E (T+E), selenium metabolism was not significantly different from that in control rats given diet T only. However, supplementation with vitamin E in the presence of adequate dietary selenium resulted in a decrease in DMSe generating activity. This effect was not observed when selenium was absent from the diet.

These results indicate that if selenium is included in a diet which is deficient in vitamin E, it will enhance its own metabolism in the liver. When vitamin E is administered, no stimulatory effect of selenium is observed. Rather, vitamin E inhibits selenium metabolism in an animal given adequate levels of dietary selenium. Thus the vitamin appears to have a modulating effect on the metabolism of selenium. The mechanism by which this occurs is not known. There are certainly many nutritional manifestations of the influence of α-tocopherol on selenium metabolism, and the ability of vitamin E to prevent or delay the onset of selenium-responsive diseases (Chapter 4), is an example of this. In addition, there are several deficiency diseases which can be prevented by the administration of either nutrient. Diplock, Baum and Lucy (1971) showed that the presence in rat liver of protein-bound selenide, which is thought to be a major source of Se for the formation of metabolically active seleno-compounds, depended upon the

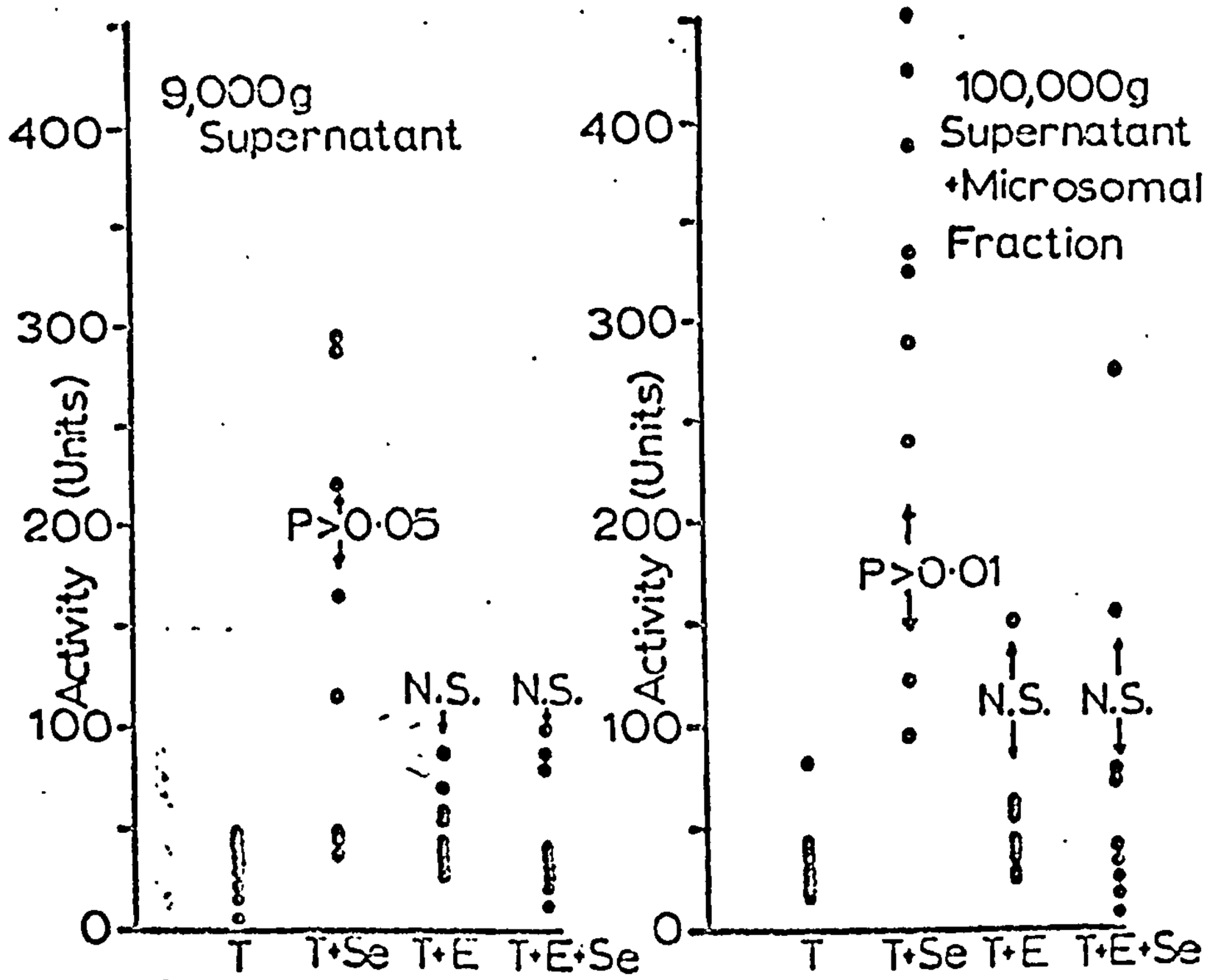


FIGURE II-I. EFFECT OF DIETARY VITAMIN E AND SELENIUM ON THE GENERATION OF DIMETHYL SELENIDE BY RAT LIVER SUBCELLULAR FRACTIONS

RATS WERE GIVEN THE VITAMIN E - AND SELENIUM - DEFICIENT TORULA YEAST DIET (T), OR THAT DIET SUPPLEMENTED WITH 0.1PPM SE (T + SE), OR 100MG/KG VITAMIN E (T + E), OR BOTH SE AND E (T + E + SE) FOR 20-26 DAYS. TECHNIQUES USED FOR PREPARATION OF FRACTIONS AND DMSE GENERATION ARE GIVEN IN THE TEXT. EACH POINT REPRESENTS A SINGLE DETERMINATION MADE ON THE LIVER OF ONE RAT. STATISTICAL EVALUATION OF THE DIFFERENCES BETWEEN THE CONTROL AND THE SUPPLEMENTED GROUPS WAS BY THE STUDENT'S TEST.

TABLE 11 - 2

DIMETHYL SELENIDE GENERATION BY RAT LIVER SUBCELLULAR FRACTIONS

	T	T + Se	T + E	T + E + Se
9000g supernatant S1	30.35 ± 13.56	107.38 ± 116.50	49.60 ± 21.54	81.03 ± 28.85
Washed microsomes Ms	47.67 ± 22.98	68.79 ± 24.70	51.12 ± 17.47	45.12 ± 11.69
Microsomes + 100,000g s't S2 + Ms	65.88 ± 70.93	251.87 ± 142.90	81.29 ± 25.19	105.39 ± 123.82

Rats were given the diets described in the text and in Fig. 11-1. Results are expressed in units defined as the percentage of the total ^{75}Se in the reaction vial which was lost during the thirty minute incubation period, expressed per g of liver; each value represents the mean + S.D. of nine determinations.

presence of vitamin E in the diet. They later suggested (Diplock and Lucy, 1973) that the biological role of vitamin E may be to stabilize membranes in vivo, partly by inhibiting peroxidation of the polyunsaturated fatty acids of membrane phospholipids, and partly by a physical interaction of the side-chains of α- tocopherol with the arachidonic acid residues of membrane phospholipids. Further, Diplock et al (1973) have suggested that the normal biosynthesis of selenide-containing proteins may require a part of the pathway proposed by Ganther (1970) for the biosynthesis of dimethyl selenide. This pathway is discussed in Chapter 8. It is suggested that the labile intermediate selenol (GSSeH), which is formed by the glutathione reductase - catalysed reduction of the selenotrisulphide GSSeSG, is the precurson of selenide in non-haem, iron-containing proteins.

The role of α-tocopherol with regard to selenium metabolism remains obscure. However, it can be argued the action of vitamin E in decreasing $DMSe$ generating activity, may serve to divert selenium from the excretory pathway to the physiological pathway when the supply of dietary selenium is limited. This control could be mediated via the microsomal membrane since, in liver, the intermediate selenol is methylated by a microsomal transmethylase (Hsieh and Ganther, 1977). and any changes in stability of the membrane are likely to be reflected in the activity of the enzyme.

CHAPTER 12

Effect of vitamin E and selenium on selenium metabolism

Parameters investigated:

(1) Generation of dimethyl selenide by 100,000g supernatant fraction of rat liver.

(2) Liver glutathione peroxidase activity in 100,000g supernatant fraction.

(3) Selenium content of 100,000g supernatant fraction.

(4) Selenium content of whole liver.

Experimental details:

Diets: The Torula yeast diet (T) has been described in Chapter 11 and its composition is given in Table 11-1. The diet is deficient both in selenium and in vitamin E and was formulated to produce dietary liver necrosis within five weeks.

Animals and feeding regimen:

Twenty-four Caesarian-derived male Wistar rats (40-50g) were obtained from a commercial breeder and divided into four groups of six. Each group was subjected to a different dietary treatment:

Dietary Regime

(a) Group T: Weanling rats were given the selenium - and vitamin E - deficient Torula yeast diet with distilled water supplied ad libitum for drinking.

(b) Group T + Se: Rats were given the basal diet T; 0.1ppm selenium as sodium selenite was added to distilled water and supplied as drinking water.

(c) Group T + E: The Torula yeast diet supplemented with 100mg DL - α - tocopherol per kg of diet was supplied to animals in this Group. Distilled water was supplied

for drinking.

(d) Group T + E + Se: The animals in this group received the vitamin E - enriched diet described in (c) above and 0.1 ppm selenium in distilled water was supplied for drinking as in (b).

Two rats from each group were killed 20, 23 and 26 days after commencement of the dietary regimes, and their livers were removed and placed in ice-cold 0.25M sucrose solution.

Homogenization and Cell Fractionation

The homogenising technique described previously was used in this and all subsequent experiments. Four passes of a conical nylon pestle of the homogenisor described by Caygill et al (1971) was routinely used to achieve reproducibility of the subcellular fractionation.

Weighed samples of liver (ca.6g) were homogenised in 20 ml 0.25M sucrose + 10^{-4}M EDTA solution. The homogenates were centrifuged for 10 min at 9000g in a MSE 18000 centrifuge. The 9000g supernatants were then centrifuged in the MSE superspeed 65 Ultracentrifuge for one hour at a speed of 100,000g and the volumes of the supernatants obtained were measured.

Each 100,000g supernatant was assayed for glutathione peroxidase activity (see Chapter 9) and the ability of the fraction to catalyse the generation of dimethyl selenide was measured as described in Chapter 8. In addition, selenium was determined by the fluorometric method in the 100,000g supernatant of rat liver and in whole liver tissue.

1. Generation of dimethyl selenide

These experiments were designed to check the results obtained from similar experiments described in Chapter 11. In both cases, the proportion of a known quantity of inorganic selenium which was converted by liver extracts to organic selenium metabolites was measured. Experiments were

described in Chapter 11 in which radioactive selenium-75 was added to inorganic selenite in tracer quantities and loss of radioactivity from the reaction tube was taken as an indication of the activity of the liver fraction. Further experimental details are given in Chapter 8. However, tracer studies using radioactive selenium in biological systems can give inaccurate results due to improper mixing of the radioactive and carrier solutions, or inhibition of the reaction under study by the decay product of the radioactive selenium. It was necessary, therefore, to assay the loss of selenium from the reaction tube by a method which did not involve the use of radioactive selenium.

The total selenium content of the reaction tube was determined by measurement of the fluorescence of the complex formed when 2, 3 - Diaminonaphthalene is added to selenite. The method used in this fluorimetric assay has been described in detail in Chapter 10, and little more will be added here. An aliquot of the reaction fluid was removed for Se-determination both before and after incubation; the difference between these results, expressed as the loss of selenium per unit volume of the reaction fluid, was related to the total volume of the fluid and could thus be expressed as the loss of selenium from the reaction tube.

Results

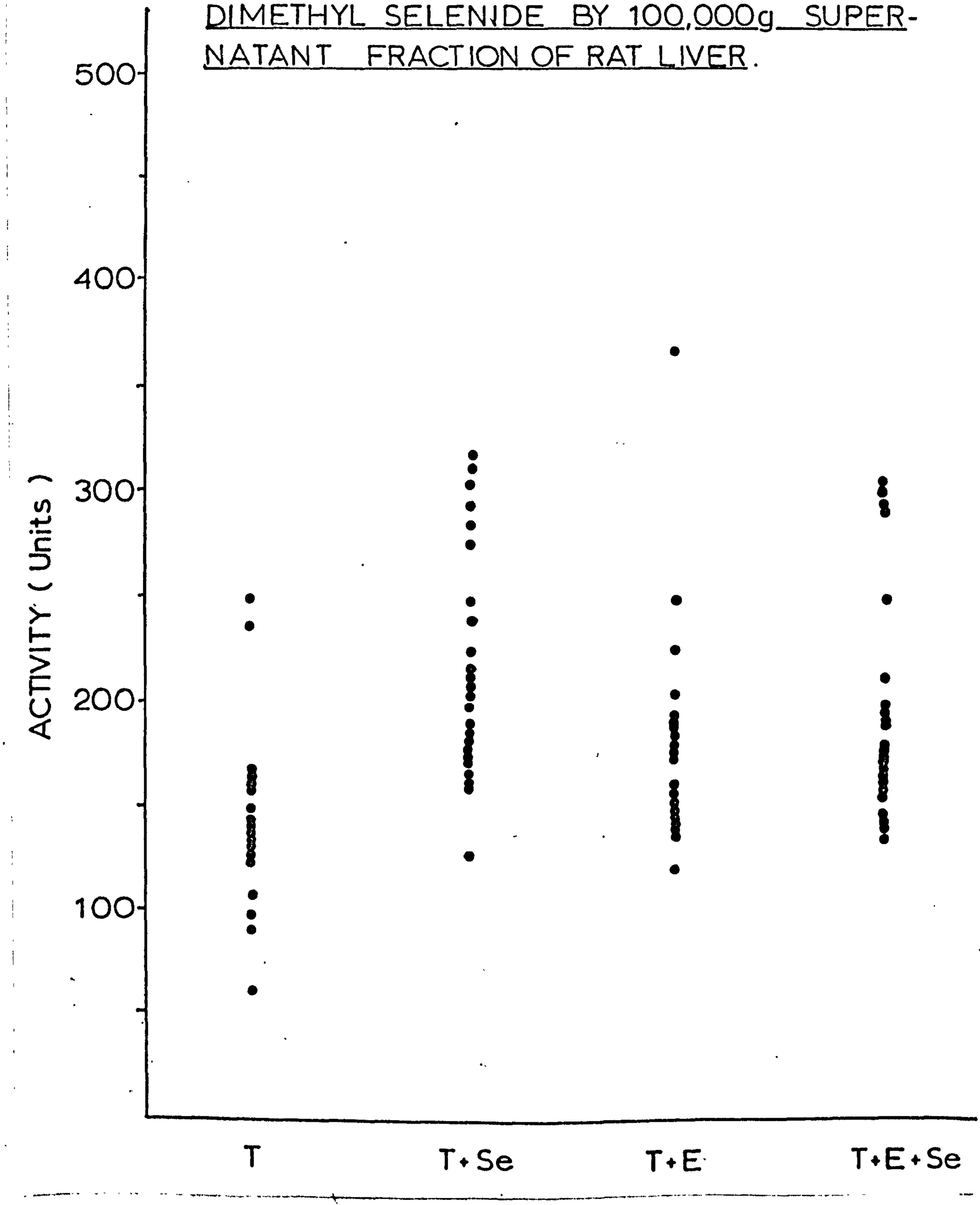
The results in Fig. 12-1 show the effect of dietary selenium and vitamin E on the generation of dimethyl selenide by rat liver 100,000g supernatant; the percentage loss of selenium was determined by fluorimetric measurement. Visual comparison with Fig. 11-1 confirms the same trend, observed in the earlier experiments, that the formation of dimethyl selenide is enhanced by prior treatment of the rats with selenium. This finding was established by statistical evaluation using the Student's t test and a significance value of $p < 0.02$ was obtained for the difference between the control group T and the selenium-supplemented group T + Se.

Fig. 12-1.

Rats were given the vitamin E- and selenium-deficient diet based on Torula yeast (T) or that diet supplemented with 0.1 ppm Se (T+Se) or 100 mg α-tocopherol per kg diet (T+E) or both selenium and vitamin E (T+E+Se) for 20-26 days. Techniques used for preparation of fractions and dimethyl selenide generation experiments are given in the text. Loss of selenium from reaction tubes was determined by fluorimetric assay. Each point represents a single determination. Six animals were used for each dietary treatment and quadruplicate determinations were made on each liver.

Activity units are defined as the percentage loss of selenium from the reaction vial expressed per g of liver. Actual loss of selenium from each vial was around 22%.

Fig. 12-1 EFFECT OF DIETARY VITAMIN E AND SELENIUM ON THE GENERATION OF DIMETHYL SELENIDE BY 100,000g SUPERNATANT FRACTION OF RAT LIVER.



Of greater importance, however, was the correlation between the range of activity units obtained using fluorimetric determination of selenium, and that obtained in ^{75}Se tracer studies. Thus, there can be no doubt that loss of trace amounts of radioactive selenium from the reaction vial was representative of the total amount of selenium lost during the incubation.

It was, therefore, decided that in subsequent experiments, ^{75}Se would be used to monitor DMSe generation, for reasons of speed, simplicity and cheapness.

2. <u>The effect of dietary vitamin E and selenium on Glutathione Peroxidase activity</u>.

The discovery by Rotruck <u>et al</u> (1973) that selenium is a component of the enzyme glutathione peroxidase (GSH-Px) has necessitated the inclusion of studies on this enzyme in the investigation of selenium metabolism.

The 100,000g supernatant of rat liver which was used in the DMSe generation experiments described in the previous section, was assayed for glutathione peroxidase activity by the coupled method of Paglia and Valentine (1967) which has been described in Chapter 9. As before, two rats from each of the four dietary groups were killed on days 20, 23 and 26 and liver fractions were prepared as previously described. The 100,000g supernatant from each liver homogenate was tested in triplicate for glutathione peroxidase activity, and the results are shown in Fig. 12-2. Units of GSH-Px activity were defined as "the number of nmoles of NADPH oxidised per minute per g liver".

<u>Results</u>:

In general, the level of glutathione peroxidase activity reflected the amount of selenium given in the diet. The increase in activity which was observed when 0.1ppm selenium was administered to the animals, was found to be dependent on the dietary level of vitamin E. When rats were

FIG. 12-2.

Effect of dietary vitamin E and selenium on liver cytosolic Glutathione Peroxidase activity.

KEY:

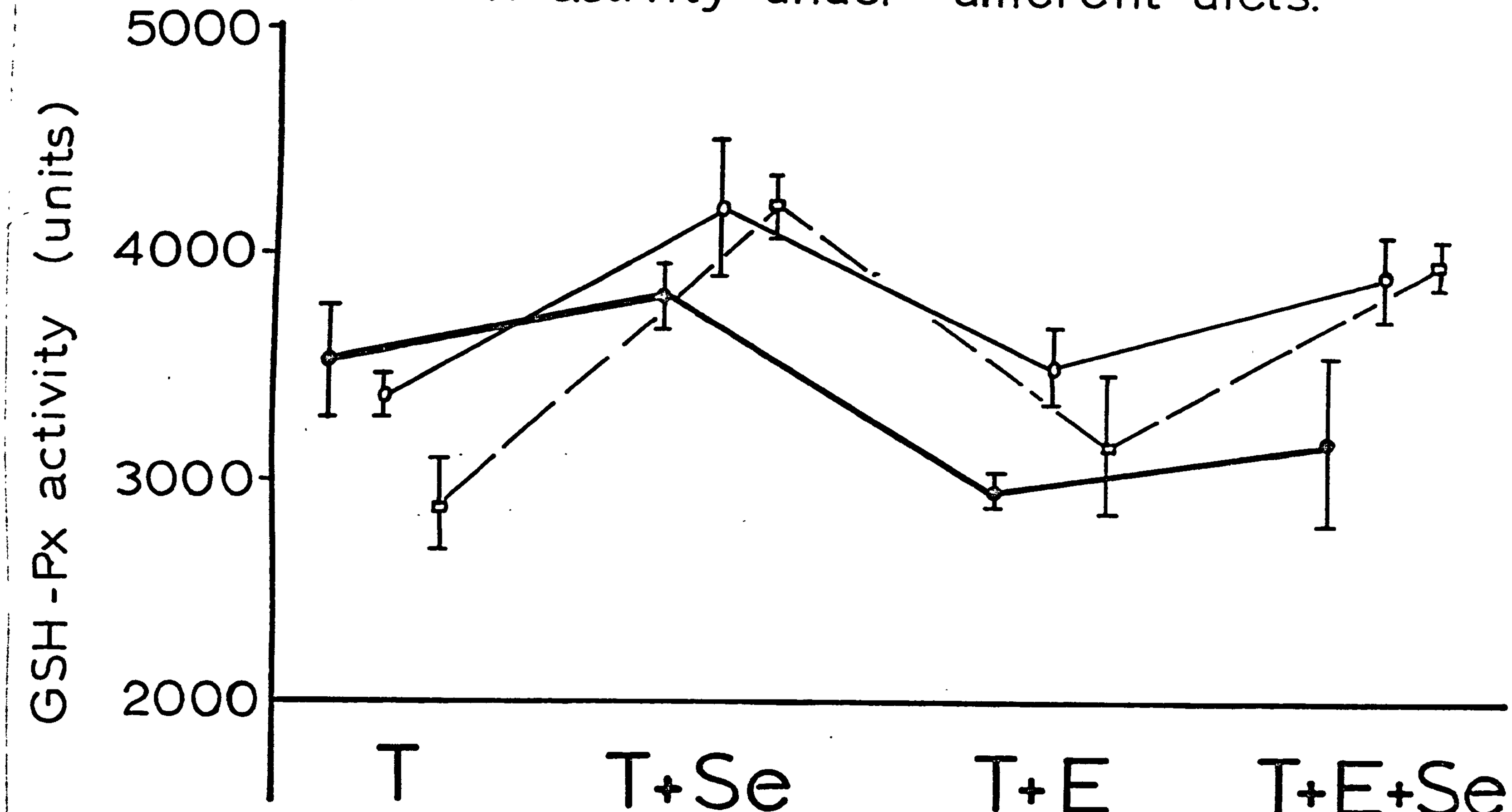
Rats killed after 20 days.

Rats killed after 23 days.


Rats killed after 26 days.

Weanling rats were given the Torula yeast diet (T) supplemented with selenium (T+Se) or vitamin E (T+E) or both (T+E+Se) for 20-26 days. Liver 100,000g supernatant was assayed for GSH-Px activity and each piont represents the mean ± S.D. of six determinations. Activity was defined as the number of n moles NADPH oxidised per minute per g liver.

GSH-Px activity under different diets.



given a vitamin E - deficient diet, the stimulatory effect of dietary selenium on GSH-Px activity increased as the period of dietary treatment increased. Thus, at 20 days, the increased liver enzyme activity of rats fed diet T + Se was not statistically different from that of rats fed diet T alone. After 23 days, however, the difference between rats in these two groups had a significance value of $p > .02$ as calculated by the Student's t test.

In rats given the selenium deficient Torula yeast diet (T), decreased liver GSH-Px activity was observed as the duration of dietary treatment increased from 20-26 days. Conversely, there was an increase in the enzyme activity of animals given 0.1 ppm selenium supplementation (T + Se). Therefore, the difference in GSH-Px activities of rats in group T and T + Se was maximal after 26 days of the dietary treatments and a significance value of $p < .001$ was established.

When vitamin E was included in the diet, supplementation with selenium (T+E+Se) for 26 days caused a smaller, but nevertheless, significant ($p < .01$) increase in glutathione peroxidase activity.

3. Analysis for Selenium in liver

(a) Liver 100,000g supernatants

The knowledge that glutathione peroxidase is a selenoenzyme and confirmation that dietary treatment with selenium stimulated the activity of this enzyme in liver cytosol led to an investigation of the selenium content of this fraction. The concentration of the complex formed by selenium and 2, 3 - Diaminonaphthalene was measured fluorimetrically and compared with values obtained using standard selenium solutions; the results are shown in Fig. 12-3.

The liver cytosolic content of selenium measured in this way did not follow the activity of GSH-Px found in the previous experiment under identical experimental conditions. Thus small and insignificant

Fig. 12-3.

Effect of dietary vitamin E and selenium on liver cytosolic selenium content.

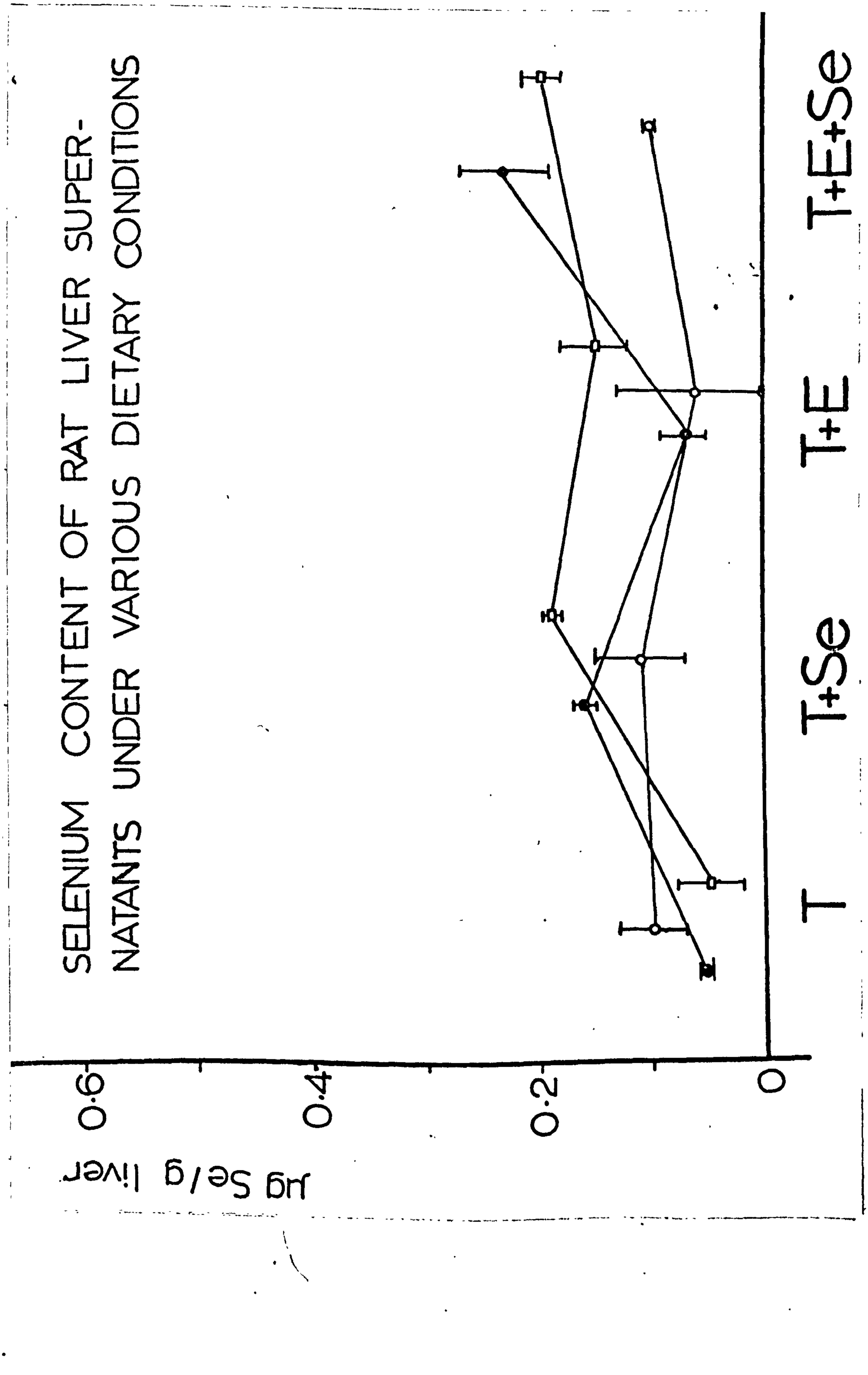
KEY:

———— Rats killed after 20 days

----O---- Rats killed after 23 days

----□---- Rats killed after 26 days

Two rats from each dietary were killed 20, 23 and 26 days after the start of the experiment. Liver 100,000g supernatant fraction was prepared (see text for details) and its selenium content was measured. Each point represents the mean ± S.D. of six determinations.


SELENIUM CONTENT OF RAT LIVER UNDER VARIOUS DIETARY CONDITIONS
NATANTS UNDER VARIOUS SUPER-
T+E+Se
T+E
T+Se
T
0.6
0.4
0.2
0
µg Se/g liver

increases were obtained when the diet was supplemented with selenium and no clear pattern of selenium depletion was observed in the liver fractions of rats given diet T only, between 20 and 26 days.

The failure to demonstrate a definite increase in the selenium content of the cytosol when 0.1ppm selenium was added to the diet could be due to contamination of the cytosol of deficient rats by selenium that was not incorporated in glutathione peroxidase. This would artificially increase the level of selenium in the control group (T) and might lead to smaller differences between control and supplemented groups.

An examination of the selenium content of whole liver was thus undertaken to determine whether selenium - or vitamin E - supplementation affected the tissue level of selenium.

(b) Wet Tissue

Samples of liver tissue were washed in 0.25M sucrose solution and blotted dry before weighing. About 0.5g was used for each determination and three samples of each liver were analysed for selenium using the techniques described in Chapter 10. The results obtained are presented in Fig. 12-4 and again the variation in liver selenium contents between 20 and 26 days of dietary treatment has been shown.

Results

As expected, the presence of selenium in the diet was reflected by an increase in the selenium content of liver; this effect was observed whether or not vitamin E was included in the diet and, when analysed by the Student's t test, was found to be highly significant. The numerical values of liver selenium content are given in Table 12-1 and each figure represents the mean $\pm$ S.D. of eighteen determinations over the three experimental days.

Fig. 12-4.

Effect of dietary vitamin E and selenium on liver selenium content.

Samples of liver tissue were taken from rats which had been given the vitamin E- and selenium-deficient Torula yeast diet (T) or that diet supplemented with 0.1 ppm Se (T+Se) or 100 mg α-tocopherol/kg diet (T+E) or both nutrients (T+E+Se) for 20 (---●---) 23 (---○---), or 26 (---□---) days. Selenium was determined by fluorimetric assay using 2,3-Diaminonaphthalene. Actual values are presented in Table 12-1.

THE EFFECT OF DIETARY VITAMIN E AND Se ON LIVER Se CONTENT.

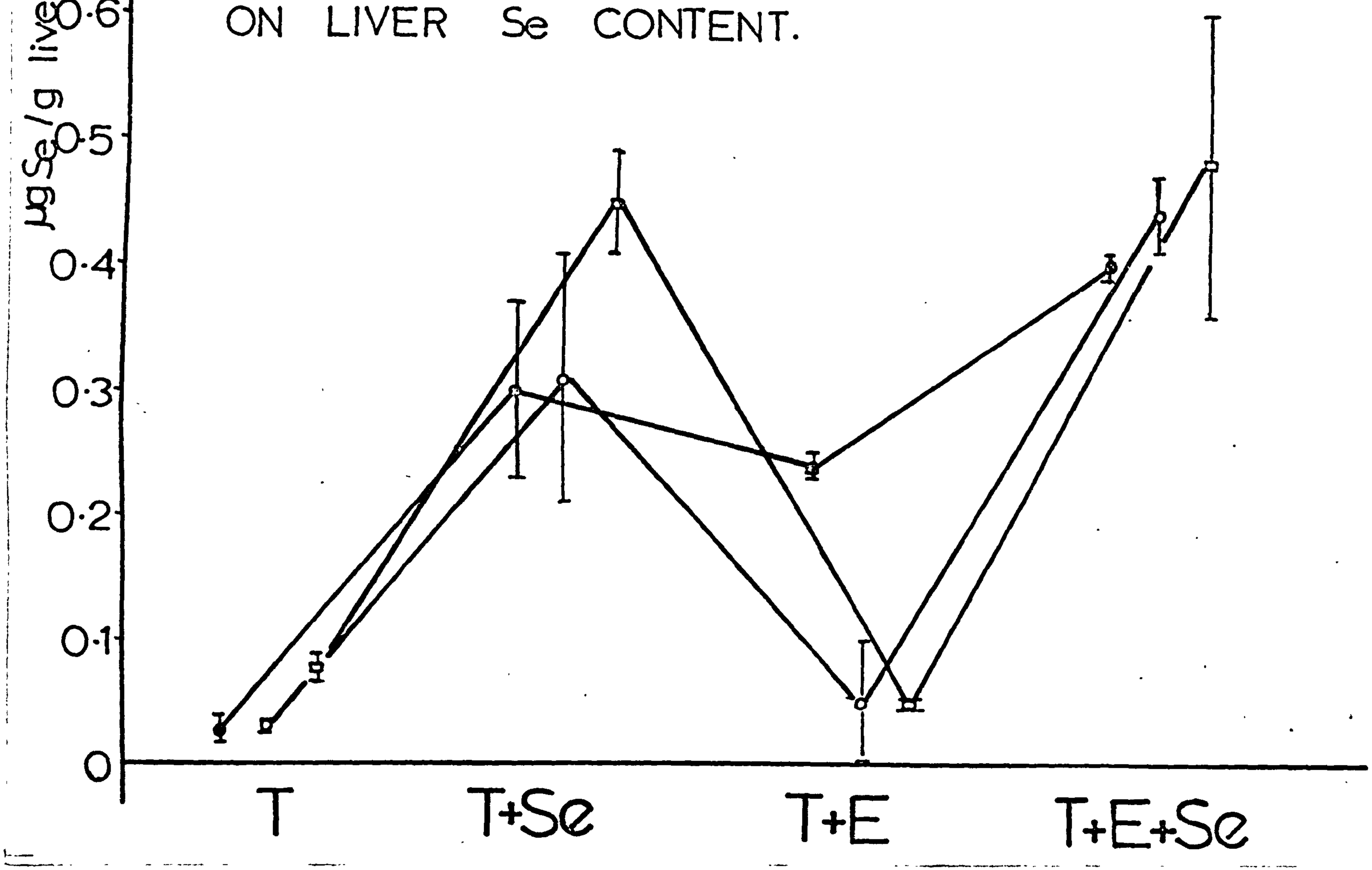


Table 12-1. Effect of dietary vitamin E and selenium on liver selenium

Selenium content of liver is expressed as μgSe per g liver (parts per million) and significance of the difference from control was established by Student's t test.

Dietary Treatment (20-26 days)	Liver selenium content (ppm) Mean ± S.D. (from 18 determinations)	Level of significance of difference from control
T	0.054 ± 0.026	
T + Se	0.351 ± 0.094	$p < .001$
T + E	0.113 ± 0.099	Not significant
T + E + Se	0.438 ± 0.065	$p \ll .001$

The inclusion of vitamin E in the diet of selenium deficient rats led to a relatively large but statistically insignificant increase in liver selenium content (Table 12-1). A possible explanation of this may be observed in Fig. 12-4; it was clear that the liver selenium of rats in group T + E was almost as high as that of rats in the selenium-supplemented group (T + Se) after only 20 days of the dietary treatments. During days 21-26, however, the liver selenium of selenium-deficient rats fell to the expected low levels; this slow rate of depletion led to a high average value for liver selenium content.

Discussion

This experiment was designed for two main reasons; one of these was to justify or condemn the use of radioactive selenium compounds as tracers in the study of dimethyl selenide generation and the other was to observe the effects, if any, of dietary treatment with vitamin E and/or selenium, on the activity of the enzyme glutathione peroxidase and to

investigate the possible correlation between GSH-Px activity and selenium levels in the liver.

The fluorimetric analysis described in Chapter 10 (which can detect 0.01μg selenium) was used to monitor the selenium content of reaction tubes used in dimethyl selenide generation experiments and the loss of selenium during the reaction was essentially similar to that obtained using ^{75}Se as tracer. This, therefore, was taken to be confirmation that, during the reaction, no distinction occurs between radioactive and unlabelled selenite and that the percentage loss obtained when radioactive selenium was used could also be taken as a measure of the total loss of selenium from the assay tube.

Measurement of glutathione peroxidase activity in the liver cytosol of rats given various dietary treatments showed that the addition of 0.1 ppm Se to the diet greatly stimulated the activity of the enzyme while the administration of dietary vitamin E had little effect on the enzyme. The effect of selenium on liver GSH-Px activity was not parallelled by increased selenium levels in the liver cytosol of rats given selenium-supplemented diets. However, when whole liver tissue was examined, significantly higher liver selenium levels were found in those rats which were given the Se-supplemented diets, with or without dietary vitamin E. In addition, in the absence of dietary selenium, the liver selenium content of vitamin E-supplemented rats was not significantly different from that of control animals.

CHAPTER 13

SILVER TOXICITY EXPERIMENT

AIM: This experiment was designed as a preliminary to those described in the next two chapters. Its aim was to examine the effect of silver and vitamin E administration on selenium deficiency and toxicity in the rat.

PROCEDURE: 80 male, weanling, Wistar rats (50-60g) were divided into ten groups of eight rats. The rats in five groups were given a basal vitamin E - deficient diet containing 8.3% Casein (Table 13-1) while the rats in the other five groups were given the same diet supplemented with 100 mg α-tocopherol per kg. diet. The animals were maintained on these diets for 18 weeks during which time their average weight had increased to 275g. and testicular atrophy had occurred. Half the rats in each group were then given 1000 ppm silver as silver acetate in their diet; at the same time, all the rats were given selenium as sodium selenite in their drinking water, at doses ranging from 0.1 to 20 ppm selenium.

Death of the animals was taken as an index of their nutritional status and the time of death and the condition of the liver at death were noted.

Table 13-2 shows the mortality of animals under the various dietary treatments; the results are presented as the ratio of the number of deaths to the total number of animals in each group. Thus, in the group given the basal vitamin E - deficient diet, supplemented with 1000 ppm silver and 0.1 ppm selenium, all four rats died. Examination of the first two columns of Table 13-2 i.e. those animals given the vitamin E - deficient diet, shows that the proportion of

TABLE 13 - 1

VITAMIN E - DEFICIENT DIET: CONTENTS

*	Casein	8.3 %
	Lard	10.0 %
	Glucose	76.0 %
	Salt Mixture	5.33 %
	Vitamin Mixture	0.4 %

* Vitamin-free casein was obtained from California Biochemicals Inc.

THE SALT MIXTURE SUPPLIED:- (g/kg)

$NaH_2PO_4 \cdot 2H_2O$	26
$CaCO_3$	18.2
KCl	3.5
Na_2CO_3	1.2
$MgSO_4 \cdot H_2O$	2.24
Ferric Citrate	0.15
$MnSO_4 \cdot 4H_2O$	0.2
$ZnSO_4 \cdot 7H_2O$	0.06
KI	0.0003
NaF	0.00025
$(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$	0.002
$CoSO_4 \cdot 7H_2O$	0.01
$Al_2(SO_4)_3 K_2SO_4 \cdot 24H_2O$	0.0007
$CuSO_4 \cdot 5H_2O$	0.02

THE VITAMIN MIXTURE SUPPLIED:- (mg/kg diet)

Vitamin A	21.4	Nicotinic Acid	90.0
Thiamine (B_1)	9	Calcium Pantothenate	90.0
Riboflavin (B_2)	19	Folic Acid	2.0
Pyridoxine (B_6)	9	Inositol	90.0
Cyanocobalamin (B_{12})	0.3	p-aminobenzoic acid	90.0
Menadione sodium bisulphite (K_3)	0.28	Choline bitartrate	900.0
Vitamin D 1.6 i.u/g			

RELATIVE NUMBER OF DEATHS IN EACH GROUP DURING Ag TOXICITY EXPT..

VIT.E ≡100mg/kg ; Ag≡1000ppm

	-E		+E	
	+Ag	-Ag	+Ag	-Ag
0.1 ppm	4/4	1/4	1/4	0/4
1.0 ppm	4/4	0/4	3/4	0/4
5 ppm	3/4	0/4	3/4	0/4
10 ppm	4/4	1/4	5/5	1/4
20 ppm	4/4	3/4	4/5	2/4

Table 13-2. Mortality of rats given the basal 8.3% Casein diet with (+E) or without (-E) 100 mg α-tocopherol per kg of diet. The number of deaths in each group is compared to the total number of animals in that group.

Silver was given in the diet while selenium was added to the drinking water.

deaths is much higher in the first column than in the second. As silver is present in the diet of rats in the first column, but not in the second, these results indicate that silver at 1000 ppm is toxic to vitamin E - deficient rats. A similar comparison of the third and fourth columns shows that silver is again toxic in the presence of vitamin E, when the dietary level of selenium is 1.0 ppm or above. In the absence of dietary silver, mortality rates are low (Columns 2 and 4) and only become significant ($p < 0.01$) when high levels of selenium (20 ppm) are administered. Immediately prior to death, these animals assumed abnormal postures with drooping heads and ears. They seemed to have difficulty in breathing and their legs were very unsteady. Post-mortem examination of their livers revealed gross reduction in size and areas of congestion, similar in appearance to dietary liver necrosis induced by feeding a diet deficient in vitamin E and selenium.

The time of survival of rats given the vitamin E - deficient diet supplemented with silver is shown in Fig. 13-1. The days of survival were counted from the commencement of silver and selenium administration. The results show that the survival period increases as the dietary level of selenium is increased, which indicated that increased levels of selenium protected the animals against silver toxicity. At 20 ppm selenium, there was a small decrease ($p > 0.05$) in the survival period, indicating again the toxicity of selenium itself.

The degree of the protection given by vitamin E against silver toxicity is indicated in Fig. 13-2. The open columns represent rats given the vitamin E - deficient diet with silver supplementation, while the closed columns represent rats given the vitamin E - and silver - supplemented diet. Significant effects of dietary vitamin E were observed only when low levels of dietary selenium were given (at 0.1 ppm Se, $p < 0.01$; at 1.0 ppm Se, $p < 0.02$). At 5 ppm selenium and above, vitamin E was not effective against silver toxicity in rats.

Fig. 13-1.

All rats were given the basal vitamin E-deficient diet for 18 weeks. From Day 1, 1000 ppm silver and various levels of selenium were administered to the animals. Results show the survival of animals from that day.

Each column represents the mean± S.D. of the result using four rats.

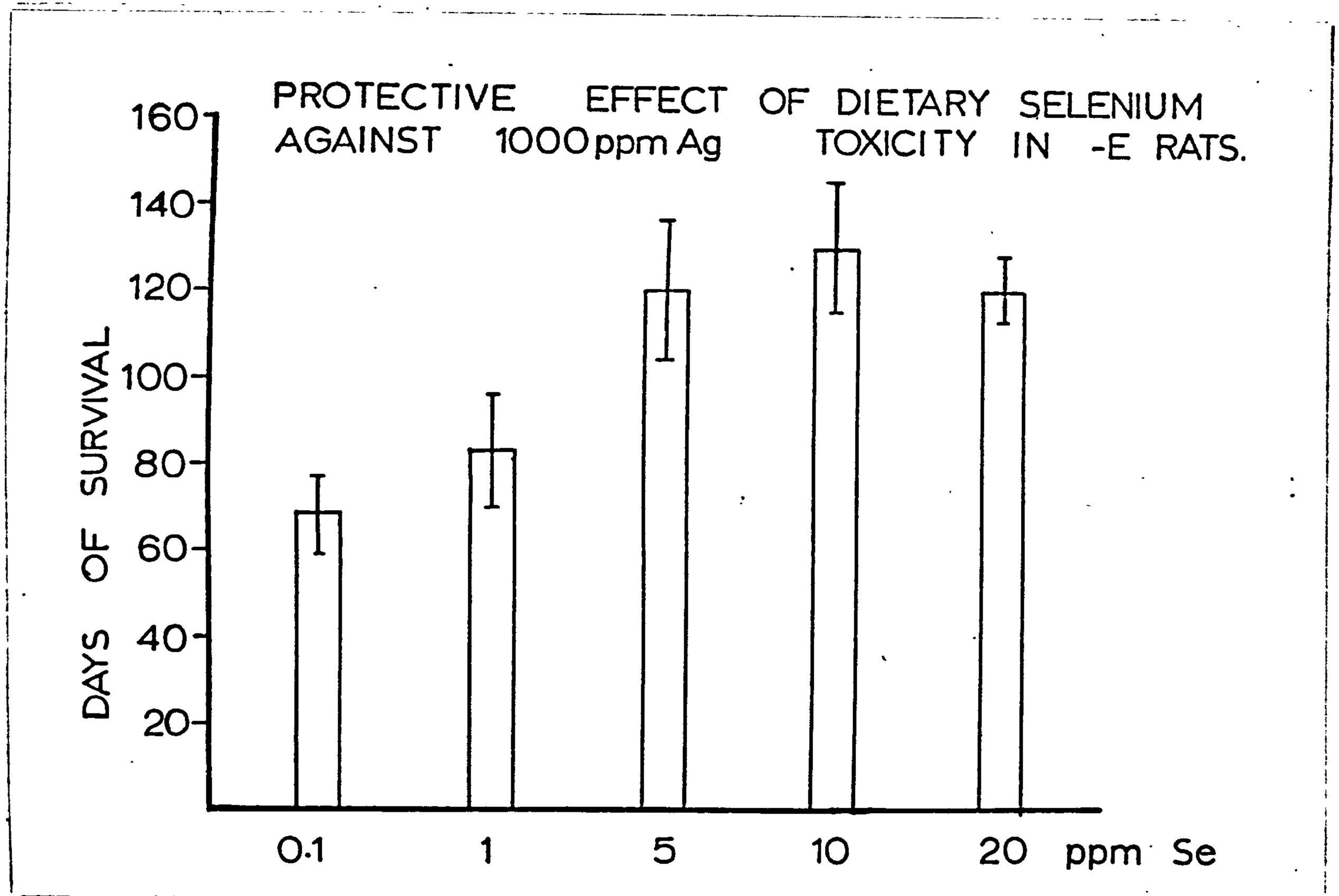


Fig. 13-2.

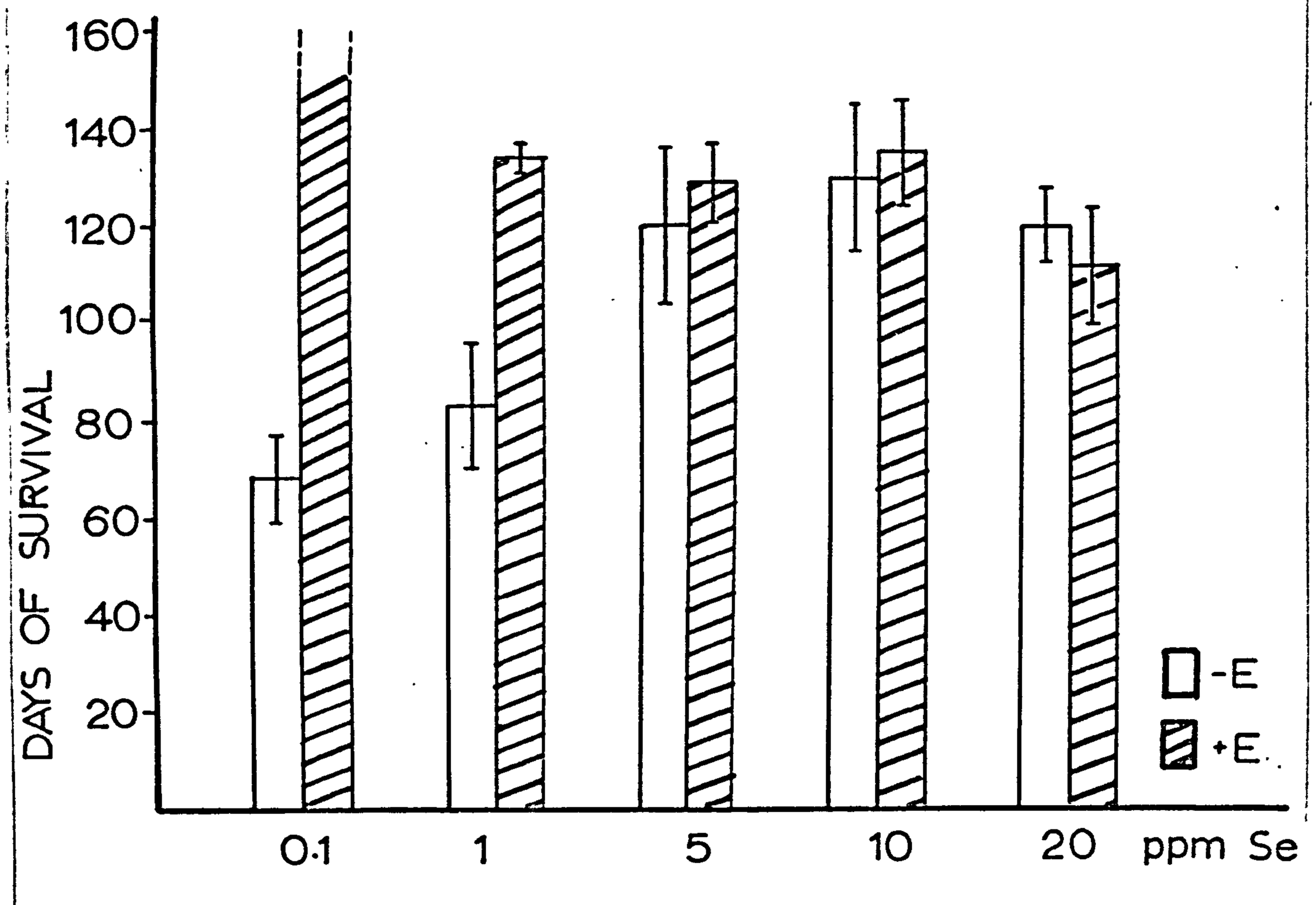
Rats were fed either the basal 8.3% Casein diet supplemented with 100 mg/kg α-tocopherol (+E)

From Day 1, 1000ppm Ag as silver acetate and selenium as Na_2SeO_3 were administered to the animals.

Each column represents the mean ± S.D. from four rats.

Note: the open-topped column for rats given vitamin E and 0.1ppm Se indicates that all the rats were alive when the experiment was terminated.

EFFECT OF VITAMIN E AT DIFFERENT LEVELS OF DIETARY SELENIUM AGAINST 1000ppm SILVER TOXICITY IN RATS.



The livers of all the animals used in this experiment were examined at the conclusion of the experiment and the recorded observations may be summarised as follows. In general, rats given the vitamin E - deficient diet and 1000 ppm silver, with either 0.1 ppm or 1.0 ppm selenium in their drinking water, showed signs of massive liver necrosis at death. The liver from one of these animals is shown in Fig. 13-3; the necrosis was superficially similar to that described by Schwarz (1958) in rats deprived of vitamin E and selenium. The necrosis appeared primarily in the central portions of the lobes, but it was never as a discreet central zone. In most cases, frank necrosis could be observed in all areas of the liver; however, in a few animals, the lesion was limited to an individual lobule.

There was, in addition, a second outstanding feature of the gross picture of dietary liver necrosis; this was the presence of haemorrhagic areas which were described by Schwarz (1958) as not being true haemorrhages, but rather accumulation and congestion of blood within the sinusoids.

Examination of the livers of animals given the same vitamin E - deficient diet plus silver, supplemented with increasing levels of selenium in the drinking water (5-20 ppm Se) revealed less prominent signs of necrosis. Congestion within the liver was observed in all the rats while necrosis was less noticeable in the high selenium groups. When the diet was also supplemented with vitamin E, necrosis was not observed in the livers of the rats, irrespective of the level of administered selenium. These livers differed from normal ones only by the presence of small haemorragic areas, which were fewer and less pronounced than those accompanying necrosis in the livers of rats given the vitamin E - deficient diet supplemented with silver. In addition, the intestines of all animals given silver had areas of fatty deposits and were grey in appearance, presumably due to the deposition of metallic silver.

As shown in Table 13-2, the majority of animals not given silver in their diet survived to the end of the experiment when they were killed and their livers were examined. As expected, the livers of these animals were normal in

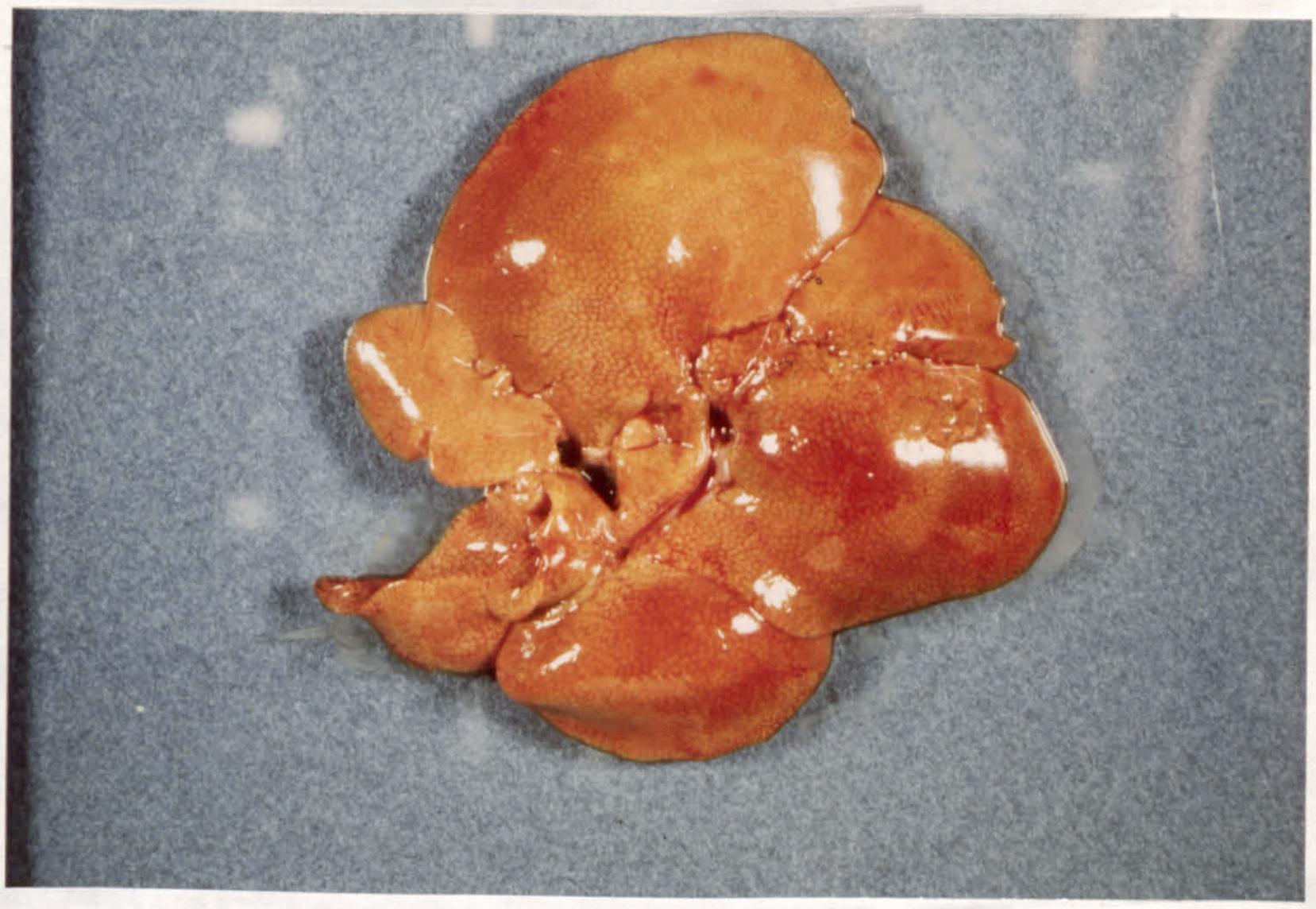


Fig. 13-3.

Appearance of the liver of a rat given a vitamin E-deficient diet, with 1000 ppm silver and 0.1 ppm selenium added as supplements.

The animal died after 63 days on this treatment and the liver was removed for examination.

appearance. When the basal vitamin E - deficient diet was given with 10 ppm selenium supplied in drinking water, only one rat died out of a group of four. Post-mortem examination showed that the liver was swollen in comparison to normal livers. Increasing the selenium level to 20 ppm resulted in increased mortality ($p < 0.01$) when three rats from a group of four, died. Examination of the livers of the dead animals showed an increase in the amount of connective tissue surrounding the liver; similarly, there were increased amounts of fatty tissue in the intestines. Rats given the basal vitamin E - deficient diet, supplemented with high levels of selenium (20 ppm) had a curious, bedraggled appearance and patchy fur a few days prior to death. These animals had laboured breathing and had difficulty in maintaining their balance; three rats in this group died within twenty days of the start of the experiment.

By contrast, however, when α-tocopherol was included in the diet, the survival period of rats given 20 ppm selenium was not uniform. Two rats given vitamin E died out of a group of four (Table 13-2) and the death of one occurred after eleven days while the other died ninety-eight days after the start of the experiment. The large difference in the time of survival suggests random mortality and no connection was made between the diets given and the deaths of the animals. Similarly, when given 10 ppm selenium and a vitamin E - supplemented diet, one rat, out of a group of four, died thirteen days after the start of the experiment; the remaining rats were still alive at the conclusion of the experiment (160 days) and it was decided that again no inference of the effect of the diet should be drawn from the isolated death. The surviving rats in both the 10 ppm and 20 ppm selenium groups which were given the vitamin E supplemented diet, all had normal appearance and healthy fur.

The results of this experiment can be summarised as follows:-

1. Silver is highly toxic in the absence of vitamin E. This was demonstrated by the high mortality ratios ($p < 0.02$) shown in

Table 13-2 when silver (1000 ppm) was administered in a vitamin E - deficient diet.

2. Vitamin E protects against silver toxicity at low levels of dietary selenium. This effect was demonstrated in Fig. 13-2 when the survival period of rats given vitamin E - deficient and vitamin E - supplemented diets were compared when silver was administered. The protective effect of vitamin E was significant at 0.1 ppm Se ($p < 0.01$) and at 1.0 ppm Se ($p < 0.02$), but was not observed at higher dietary selenium levels.

3. Increasing levels of selenium protect against silver toxicity in the absence of vitamin E. Fig. 13-1 shows increased survival periods ($p < 0.01$ at 10 ppm Se) as the level of dietary selenium was increased.

4. Selenium at 20 ppm is toxic in the absence of vitamin E and vitamin E protects against this toxicity. The administration of high levels of selenium, whether or not silver was included in the diet, resulted in increased mortality ratios. Fig. 13-1 shows an indication which was confirmed in subsequent experiments, that 20 ppm selenium, given in the presence of dietary silver, caused shorter survival. In the absence of silver, nearly all rats given the vitamin E - deficient diet and between 0.1 and 10 ppm selenium survived until the end of the experiment while of those given 20 ppm selenium, a 75% mortality rate within 20 days of the start of the experiment was observed. The greatly increased survival period when α- tocopherol was included in the diet, confirmed that vitamin E protects against high selenium toxicity.

This experiment confirmed the observation of earlier workers (see Chapter 7) that there is an interaction between silver and selenium, and between these

elements and vitamin E; it remains, however, to determine the nature of this relationship and the following three chapters of this thesis are concerned with examining the effects of selenium, vitamin E and silver on selenium metabolism.

CHAPTER 14

The Effect of dietary selenium and silver on selenium metabolism in vitamin E - deficient rats.

Parameters investigated

1. Generation of dimethyl selenide by a 100,000g supernatant fraction of rat liver.

2. Liver glutathione peroxidase activity in a 100,000g supernatant fraction.

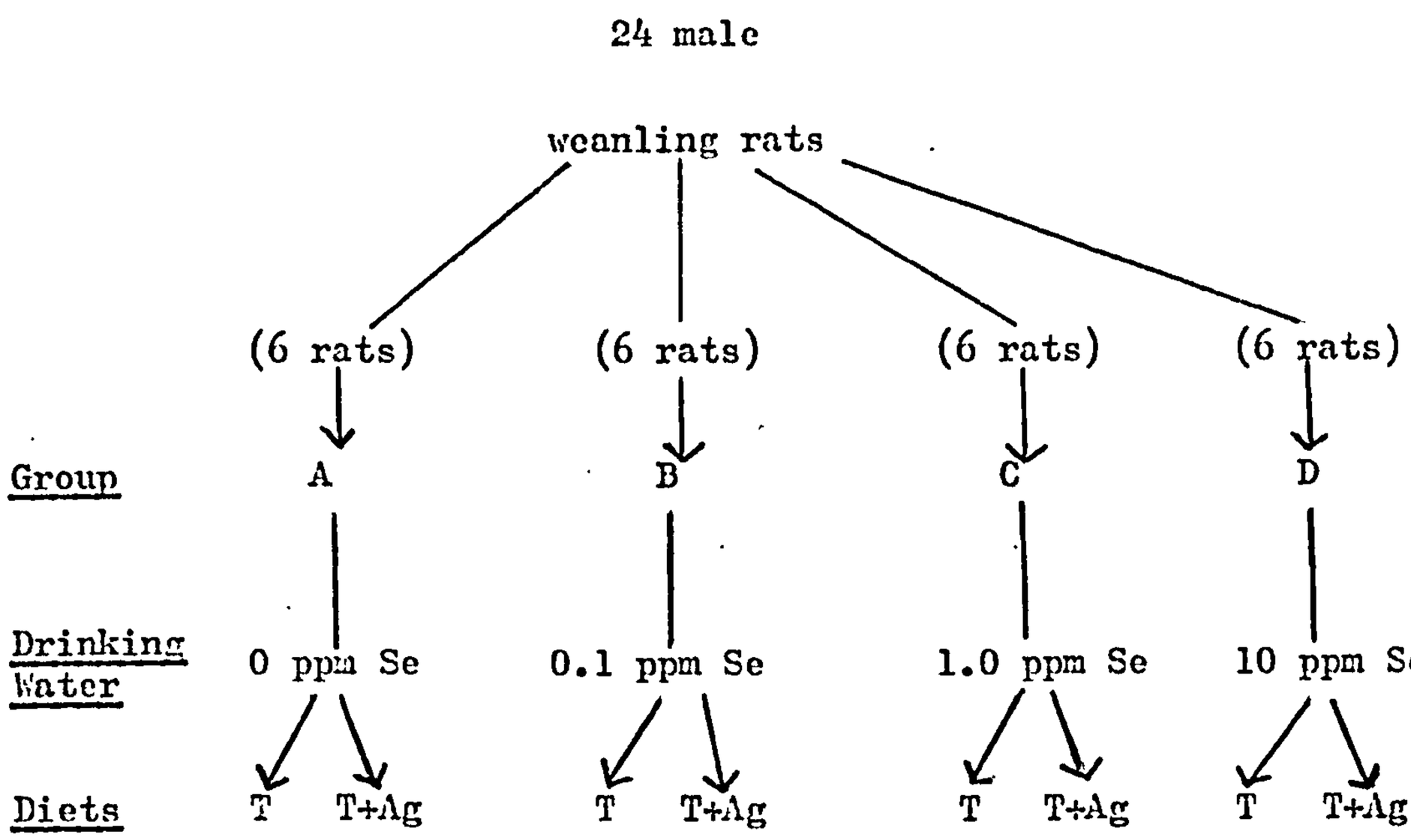
3. Selenium content of the liver.

Experimental details

Diets: The selenium - and vitamin E - deficient Torula yeast diet (T) has been described in Chapter 11 and a list of its contents is given in Table 11-1. Supplementary selenium as sodium selenite was administered in the drinking water of the animals, and silver as silver acetate was mixed with the powdered diet, in the amounts given in the experimental protocols.

Animals and Feeding Regimen:

Twenty-four male, weanling Wistar rats (40-50g) were purchased and divided at random into four groups of six. Half the animals in each group were given the Torula yeast diet T while the others were fed the same diet supplemented with 500 mg silver (as silver acetate) per kilogram of diet (T + Ag). Each group was then given a different level of selenium in drinking water, ranging from 0 ppm Se (Group A) to 10 ppm Se (Group D). The dietary treatments are summarised below:



Two rats from each group (one given diet T and the other given the silver-supplemented diet, T + Ag) were killed 21, 22 and 23 days after the start of the experiment.

Preparation of Liver Extracts

A 30% homogenate of each liver was prepared in 0.25 M sucrose solution, and the 100,000g supernatant fraction was obtained as previously described. This fraction was used in dimethyl selenide generation experiments and was also tested for glutathione peroxidase activity.

I. Dimethyl Selenide Generation

The effect of silver and different levels of dietary selenium on dimethyl selenide synthesis and thus on selenium metabolism, was investigated. The 100,000g supernatant fraction from each liver was tested in quadruplicate for its ability to synthesise dimethyl selenide. Details of the experimental procedure have been given in Chapter 8.

Results:

Activity of liver supernatant fractions in dimethyl selenide generation experiments has been calculated in terms of the loss of ^{75}Se

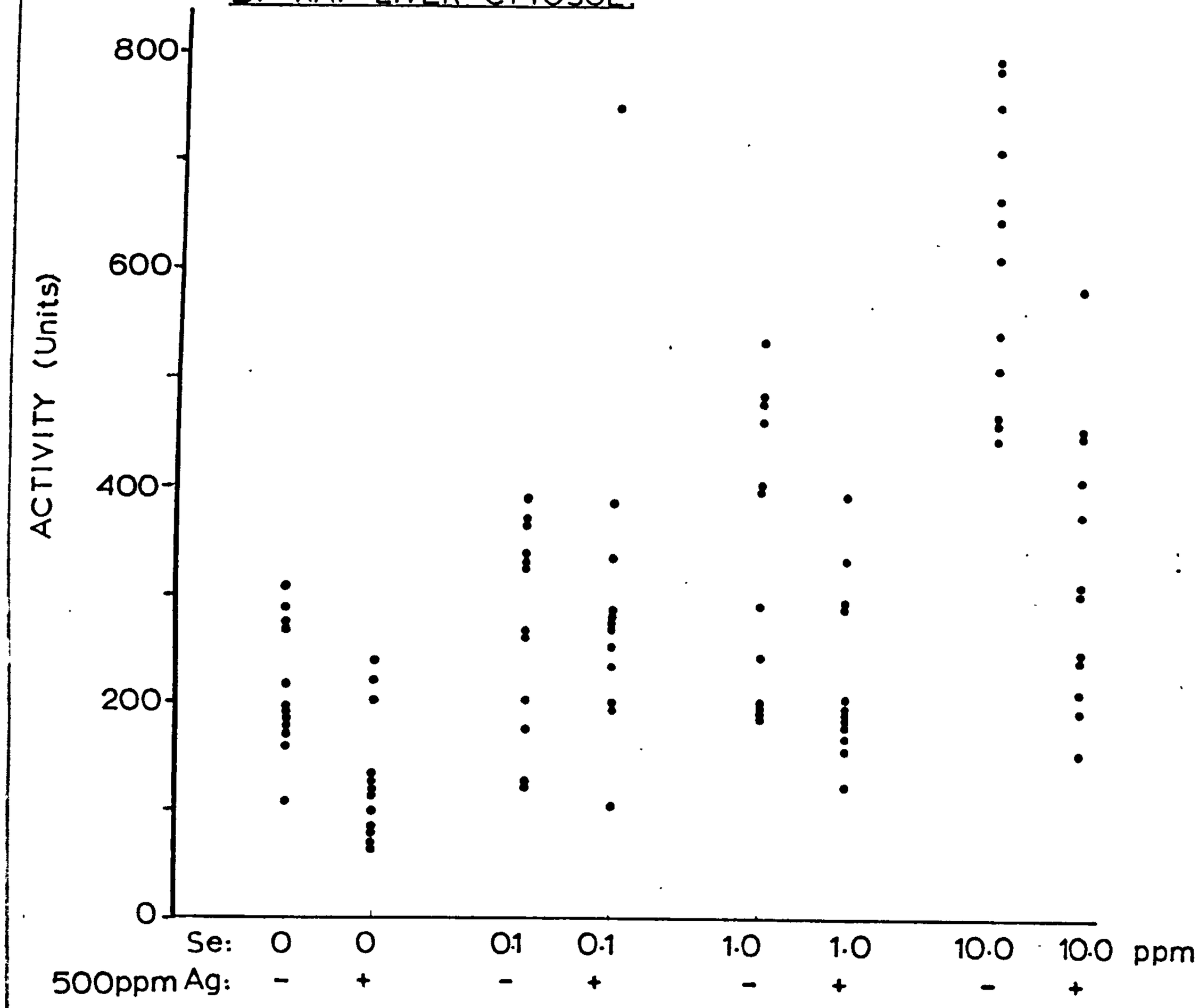
Fig. 14-1.

24 male weanling rats (40-50 g) were given the vitamin E- and selenium-deficient Torula yeast diet; half of the rats received in addition, 500ppm silver in their diet. The animals were then divided into four groups, one of which received no selenium supplement. Rats in the other three groups were given 0.1ppm, 1.0ppm and 10ppm selenium in their drinking water.

Animals were killed 21-23 days after commencement of the dietary treatments and 100,000g supernatant fractions of liver homogenates were prepared. The supernatant fraction from each liver was tested in quadruplicate for its ability to generate dimethyl selenide. Each point represents a single determination made on the liver of one rat.

Units of activity are defined as the percentage loss of selenium per g of liver.

Fig.14-1 THE EFFECT OF DIETARY Se AND Ag ON DMSe GENERATION BY RAT LIVER CYTOSOL



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from the reaction tube during the incubation period, and results are presented in Fig. 14-1.

Effect of Selenium

Visual appraisal of Fig. 14-1 shows that the effect of increasing levels of dietary selenium is to increase the activity of liver cytosol in DMSe generation. However, statistical analysis by the Student's t test showed that only those rats given 10 ppm selenium (without silver) in their diets could synthesize dimethylselenide at a rate which was significantly higher ($p<.01$) than that of control rats in group A.

Effect of Silver

The general trend in the effect of silver on DMSe generation is to decrease the activity of liver extracts at all the selenium levels investigated. However, the effect of silver increases with increasing level of dietary selenium and at 10 ppm Se, silver decreased DMSe generation by a significant amount ($p<.02$).

II. Effect of dietary selenium and silver on glutathione peroxidase activity

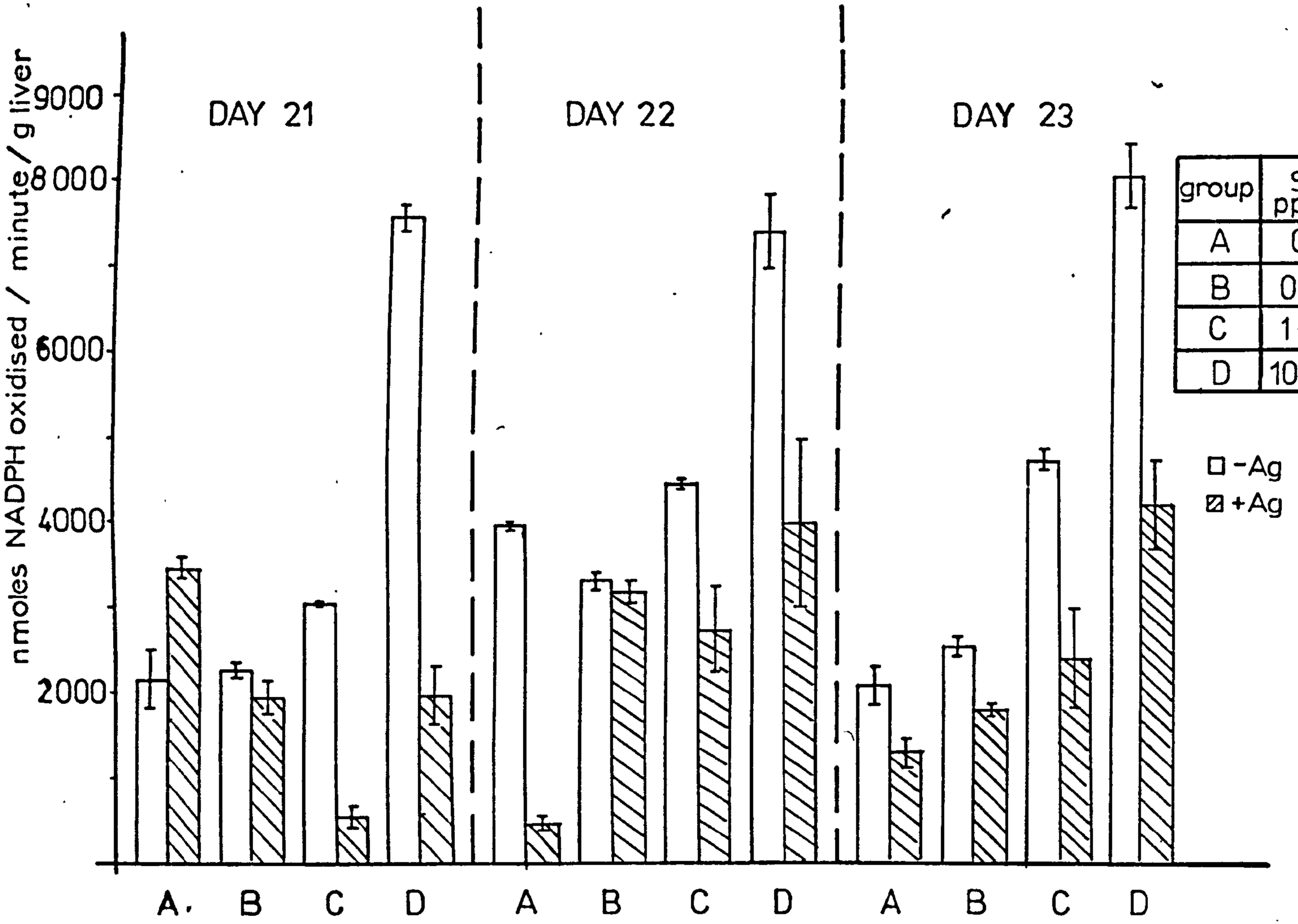
Triplicate analyses of the GSH-Px activity in the liver 100,000g supernatant fraction from each rat were carried out and the results are presented in Fig. 14-2. The open bars represent those rats not given silver in their diet while the animals represented by closed bars were given 500 ppm silver. If each day is considered separately, the level of enzyme activity in the absence of silver (open bars) generally increased with increasing amount of dietary selenium administered to the animals. Thus, those animals in group D which were not given silver had the highest level of GSH-Px activity. This gradation is clearer at 23 days of dietary treatment by which time it would be expected that rats given 0ppm selenium (group A) would be deficient in the element and thus have low enzyme activities,

Fig. 14-2.

Rats were given the selenium- and vitamin E-deficient Torula yeast diet (group A) or that diet supplemented with 0.1ppm selenium (B), 1.0ppm Se (C) or 10ppm selenium (D) as sodium selenite in their drinking water. In addition, half the rats in each group were given 500ppm silver as silver acetate in their diet. Animals were killed 21, 22 and 23 days after the start of the experiment and glutathione peroxidase activities in 100,000g supernatant fractions of liver homogenates were measured.

Each bar represents the mean ± S.D of three determinations.

Fig.14-2: EFFECT OF DIETARY SELENIUM AND SILVER ON GLUTATHIONE PEROXIDASE ACTIVITY IN VIT.E DEFICIENT RATS.



group	Se ppm
A	0
B	0·1
C	1·0
D	10·0

while those fed 10ppm Se should have high activities. Thus the effect of dietary selenium is clearly to increase glutathione peroxidase activity.

Addition of silver to the diet of vitamin E-deficient rats exerted an influence on glutathione peroxidase activity which was more pronounced at higher levels of dietary selenium. The enzyme activity of rats in groups C and D (given 1.0 and 10 ppm Se respectively) was significantly lowered ($p<.001$) by the addition of 500 ppm silver to the diet while the rats in groups A and B (0 and 0.1 ppm Se) showed less response to dietary silver. Although the general effect of silver in the latter groups was to lower GSH-Px activity, the decrease was less than in groups C and D; silver in fact increased the activity of the enzyme ($p<.05$) in selenium deficient rats (groupA) after 21 days of dietary treatment.

Thus, although glutathione peroxidase activity increased with increasing levels of dietary selenium, the addition of silver to the diet resulted in a decrease in enzyme activity and this effect was greater at higher selenium levels.

III. Effect of dietary selenium and silver on the selenium content of rat liver

Liver from each rat was washed with 0.25M sucrose solution and blotted dry with tissue paper. Three ca. 0.5g samples were then analysed for selenium using the fluorimetric assay discussed in Chapter 10 and results were expressed as μg Se per g liver (parts per million).

Effect of Se

The amount of selenium found in rat liver was greatly dependent on the dietary level which was administered to the animal (Fig. 14-3). Supplementation of the diet with increasing amounts of selenium led to increased levels of the element found in liver, and

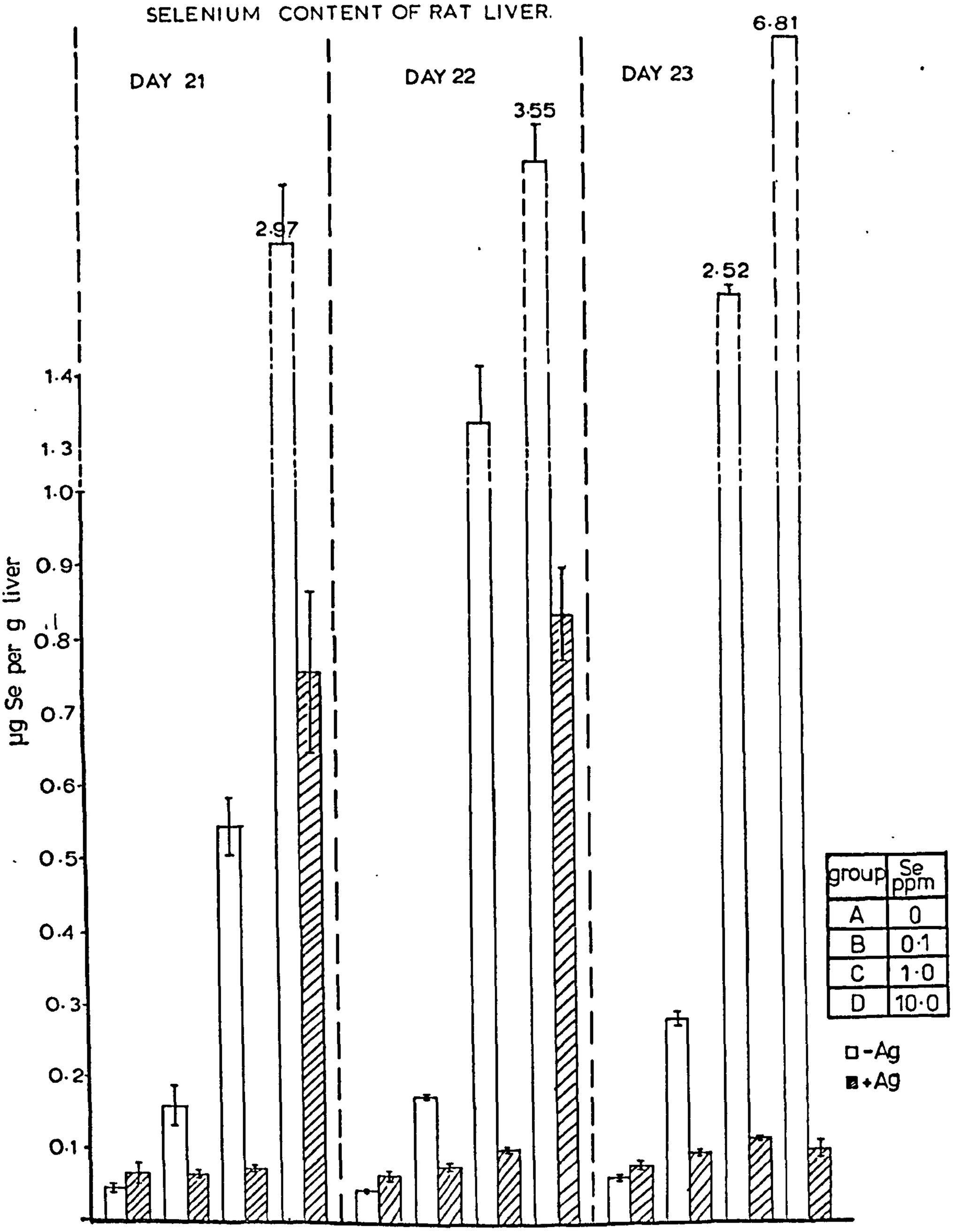


Fig. 14-3. Male weanling Wistar rats (40-50g) were given the selenium- and vitamin E-deficient diet (group A) or that diet supplemented with 0.1ppm Se (B), 1.0ppm Se (C) or 10ppm Se (D) as sodium selenite in their drinking water. Half the rats in each group were also given 500ppm silver as silver acetate in the diet. Rats were killed 21, 22 and 23 days after the commencement of the dietary regimes and samples of liver were washed, weighed and analysed for selenium using the 2,3-Diaminonaphthalene flourimetric method. Each bar represents the mean± S.D of three determinations.

the significance of the difference from control animals was established statistically using the Student's t test : for rats given 0.1 ppm selenium $p<.02$. Higher selenium levels in the diet resulted in larger statistical differences from the control animals; thus $p<.001$ for rats given 1.0 and 10 ppm selenium.

Effect of Ag

The overall effect of silver was to lower the selenium content of the liver of rats given 0.1 to 10 ppm selenium in their drinking water. Animals in group A which were selenium-deficient and thus had low selenium levels, showed a slight increase in liver selenium when silver was added to the diet. The increase was small however and of no statistical significance; it should be stressed that only those rats in group A which were not given any dietary selenium showed this effect with silver. In all the other rats which had received selenium levels ranging from 0.1 ppm to 10 ppm Se, the addition of 500 ppm silver to the diet caused a decrease in the selenium content of liver which was analysed by the Student's t test; the significance value of the effect of silver ranged from $p<.01$ when the level of dietary selenium was 0.1 ppm, to $p<<.001$ at 10 ppm Se.

IV. Discussion

It is not known precisely how silver interacts with selenium but it is thought (Diplock et al, 1967) that silver complexes free selenium in the tissues. The experiment described in Chapter 13 showed that silver protects against selenium toxicity and such an effect could be explained by the removal of selenium, possibly in the form of selenide, by silver. Analyses of the selenium content of liver showed (Fig. 14-3) that, in the presence of dietary silver, the level of selenium detected by the fluorimetric assay was significantly decreased when the diet contained normal or high levels of selenium. Thus the

amount of free selenium in the tissues was much lower in the presence of silver and was therefore much less toxic; hence the longer survival period and healthier appearance of those animals given silver in addition to toxic doses of selenium (Chapter 13).

Examination of another index of selenium in the tissues, the activity of glutathione peroxidase, showed (Fig. 14-2) that the effect of silver was to decrease enzyme activity and again this effect was more pronounced at the higher, more toxic, levels of dietary selenium. Increasing levels of dietary selenium increased the activity of the enzyme in the absence of silver, presumably as a result of increased availability of selenium which can be incorporated into the enzyme. It would therefore be expected that the action of silver in removing free selenium would result in a decrease in enzyme activity in the presence of dietary silver. This effect was observed in Fig. 14-2.

Finally, when the pathway for the detoxyfication of acute doses of selenium was examined (Fig. 14-1), again the effect of silver was greatest at the highest level of dietary selenium which was used in this experiment. The synthesis of dimethyl selenide is a protective route in tissues for the removal of toxic amounts of selenium. Experiments reported in Chapters 11 and 12 showed that dietary selenium has an inductive effect on this protective pathway. If silver protects against selenium toxicity in tissues by the removal of free selenium, then the tissue would "see" less selenium than is actually present and thus moderate the activity of the DMSe pathway to cope only with the amount of free selenium. This effect was observed in Fig. 14-1.

CHAPTER 15

The Effect of dietary vitamin E, selenium and silver on selenium metabolism

Parameters Investigated:

(1) Generation of dimethyl selenide by the 100,000g supernatant fraction of rat liver.

(2) Liver glutathione peroxidase activity in the 100,000g supernatant fraction.

(3) Selenium content of liver tissue.

Experimental details:

Diets: The basal diet given to the rats was the selenium - and vitamin E - deficient Torula yeast diet (T) which has been described in Chapter 11. Dietary supplements of silver at 1000 ppm Ag as silver acetate, and vitamin E at 100 mg DL -α- tocopherol per kg were mixed into the diet. The level of silver used was double that in the previous experiments described in Chapter 14 in order to investigate the effects of a higher level of the toxic compound on the parameters listed above, and also to correspond with the experimental conditions used in Chapter 13.

Selenium supplements of 0.1 ppm to 10 ppm Se were administered as sodium selenite added to drinking water.

Animals and feeding regimen:

Forty-eight male, weanling wistar rats (40 - 60g) were divided into eight groups of six. The animals in four of these groups were given the basal Torula yeast diet (T) while those in the other four groups received the same diet supplemented with α- tocopherol (T+E). Half of the animals in each group were given 1000 ppm silver as silver acetate and all the animals received different levels of dietary selenium, ranging from 0 to 10 ppm selenium. The grouping and dietary treatments of the experimental animals is summarized in Table 15 - 1.

Table 15 - 1

Feeding regimen in the experiment to investigate the effect of dietary vitamin E. selenium and silver on selenium metabolism in rats.

	Se (ppm)	1000 ppm Ag	Group
48 weanling rats -E	0	-	A
		+	
	0.1	-	B
		+	
	1.0	-	C
		+	
	10	-	D
		+	
48 weanling rats +E	0	-	A^+
		+	
	0.1	-	B^+
		+	
	1.0	-	C^+
		+	
	10	-	D^+
		+	

Rats were killed 20, 23 and 26 days after commencement of the diets and their livers were immediately placed in ice-cold 0.25M sucrose solution.

Preparation of Liver fractions:

A homogenate containing 3 parts liver and 7 parts sucrose solution was prepared using the homogenisor described previously. The homogenate was centrifuged for ten minutes at 9,000g in a MSE 18000 centrifuge. The post-mitochondrial supernatant fraction was decanted, its volume measured and it was centrifuged at 100,000g for one hour in a MSE Superspeed 65 ultra-centrifuge. The cytosol or post-microsomal supernatant fraction thus obtained, effectively contains the soluble enzymes from the cell matrix without the membranous structures and was used in the dimethylselenide generation experiments and was also assayed for glutathione peroxidase activity.

I. Generation of dimethyl selenide:

Liver extracts from rats which had been given dietary supplements of vitamin E, selenium and silver either singly or in combination, were tested for their ability to synthesize dimethyl selenide. The 100,000g liver supernatant fraction from each rat was analysed in quadruplicate after 20 - 26 days of dietary treatment; details of the experimental procedure for examining DMSe synthesis are given in Chapter 8.

Results:

The effects of dietary selenium and silver on the generation of dimethyl selenide by liver cytosol from vitamin E-supplemented rats are shown in Fig. 15 - 1. As the dietary level of selenium was increased from zero to 10 ppm, the ability to liver extracts to catalyse the synthesis of dimethyl selenide also increased and a very high level of activity was recorded for those animals given 10 ppm selenium. Administration of 1000 ppm silver effected a decrease in the ability of liver cytosol to synthesize DMSe and this effect was greatest at a selenium dietary level of 10 ppm.

Fig. 15-1.

The Effect of Dietary Selenium and Silver on the Generation of Dimethyl Selenide by rat liver extracts

48 weanling rats were given the basal Torula yeast diet supplemented with 100 mg α-tocopherol per kg diet (T+E) and they were divided into four groups. Half the animals in each group received 1000ppm silver as silver acetate in their diet and each group was given a different level of dietary selenium ranging from zero to 10ppm. Animals were killed 20-26 days after commencement of the dietary treatment. Rat liver 100,000g supernatant fraction was used in DMSe generation experiments and a unit of activity was defined as the percentage of total ^{75}Se lost from the reaction tube, per g of liver.

Statistical evaluation was by the Student's t test and results indicate the significance of the difference from selenium deficient rats.

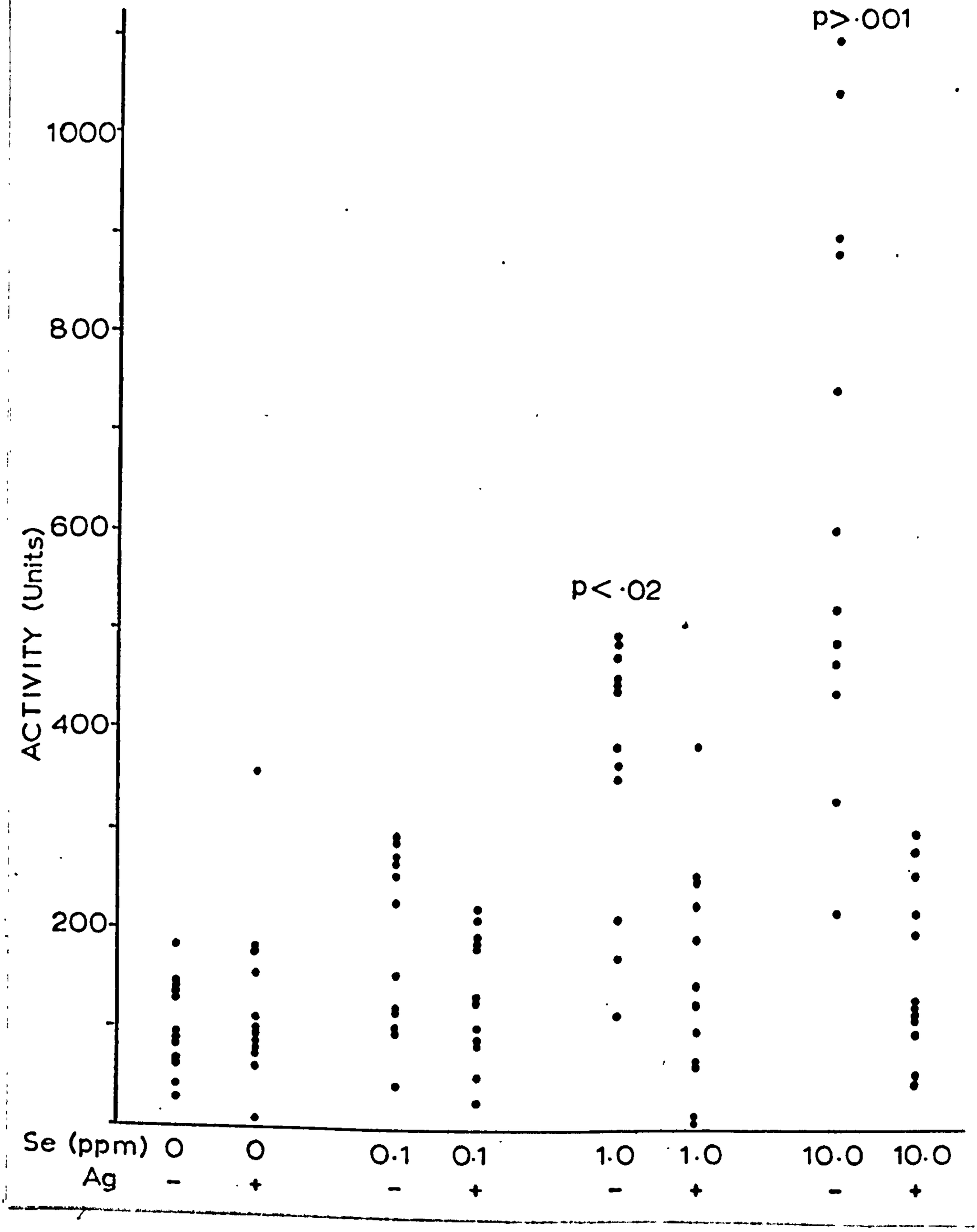

p>·001
p<·02
1000
800
600
400
200
ACTIVITY (Units)
Se (ppm) 0 0 0.1 0.1 1.0 1.0 10.0 10.0
Ag - + - + - + - +

TABLE 15 - 2

The Effect of dietary supplementation with vitamin E (100 mg / kg diet), silver (1000 ppm as silver acetate) and selenium at levels ranging from 0.1 to 10 ppm on the generation of dimethylselenide by rat liver cytosol.

Selenium (ppm)	Silver (1000 ppm)	ACTIVITY OF LIVER 100,000g SUPERNATANT FRACTION IN DMSe GENERATION (UNITS*)	
		-E	+E
0	-	116.4 ± 31.3	101.1 ± 44.2
	+	95.9 ± 34.3	123.5 ± 83.4
0.1	-	130.9 ± 38.0	185.2 ± 84.6
	+	121.9 ± 23.8	133.8 ± 61.5
1.0	-	163.6 ± 28.7	365.0 ± 124.3
	+	125.5 ± 35.9	153.0 ± 106.7
10.0	-	221.6 ± 62.9	643.3 ± 274.0
	+	109.5 ± 32.1	159.3 ± 82.9

* A unit of activity is defined as the percentage loss of ^{75}Se from the incubation tube expressed per gram of liver.

Figures represent the mean ± S.D. of twelve determinations.

The results presented in Fig. 15 - 1 represent those animals which were given dietary supplements of vitamin E; the effect of dietary selenium and silver on the vitamin E - deficient rats was similar to that obtained in the last Chapter (Fig. 14 - 1) using experimental conditions which were identical to those used here except that a lower level of silver supplementation was employed. The actual activities of the liver fractions from both vitamin E - deficient and supplemented rats are given in Table 15 - 2. In general, the activity of liver extracts from vitamin E - deficient rats was lower than that from vitamin E - supplemented rats.

II. Glutathione Peroxidase Activity:

The 100,000g supernatant fraction of liver from each of the experimental animals was analysed in triplicate to determine the effects of dietary selenium, silver and vitamin E on the activity of glutathione peroxidase in the liver.

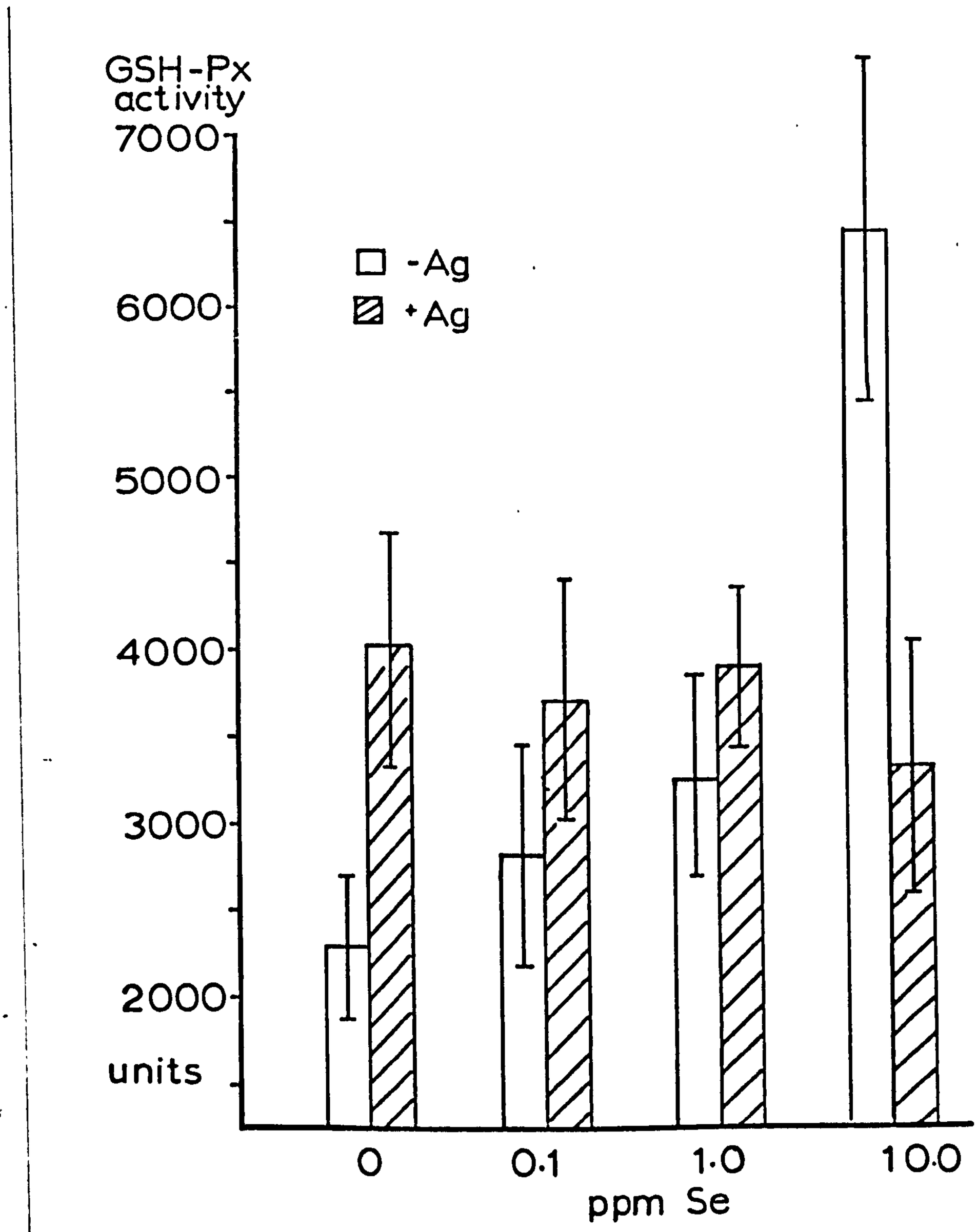
Figure 15 - 2 shows the effect of dietary supplementation with silver and selenium on liver GSH - Px activity in rats fed adequate levels of vitamin E. Increasing levels of dietary selenium administered in the absence of silver (open bars in Fig. 15 - 2) led to increased activity of the enzyme up to the highest level of selenium studied. The influence of silver on liver glutathione peroxidase in the presence of dietary vitamin E was varied and depended on the level of dietary selenium administered to the animal. At zero, 0.1 and 1.0 ppm Se, dietary silver increased liver GSH-Px activity but at 10 ppm Se, the addition of 1000 ppm silver to the diet decreased the activity of the enzyme by about 65%.

The effect of vitamin E on liver cytosolic glutathione peroxidase is shown in Fig. 15 - 3. The closed bars represent those animals which received 100 mg α-tocopherol / kg diet and liver enzyme activities of

Fig. 15-2.

Rats were given the basal Torula yeast diet supplemented with 100mg α-tocopherol per kg diet. Half the rats also received 1000ppm silver as silver acetate and selenium, where given, was added as sodium selenite to drinking water. Rats were maintained on diets for 20-26 days after which they were killed and 100,000g supernatant of their livers were prepared and used in GSH-Px assays.

Each bar represents the mean $\pm$ S.D of triplicate determinations on three livers.



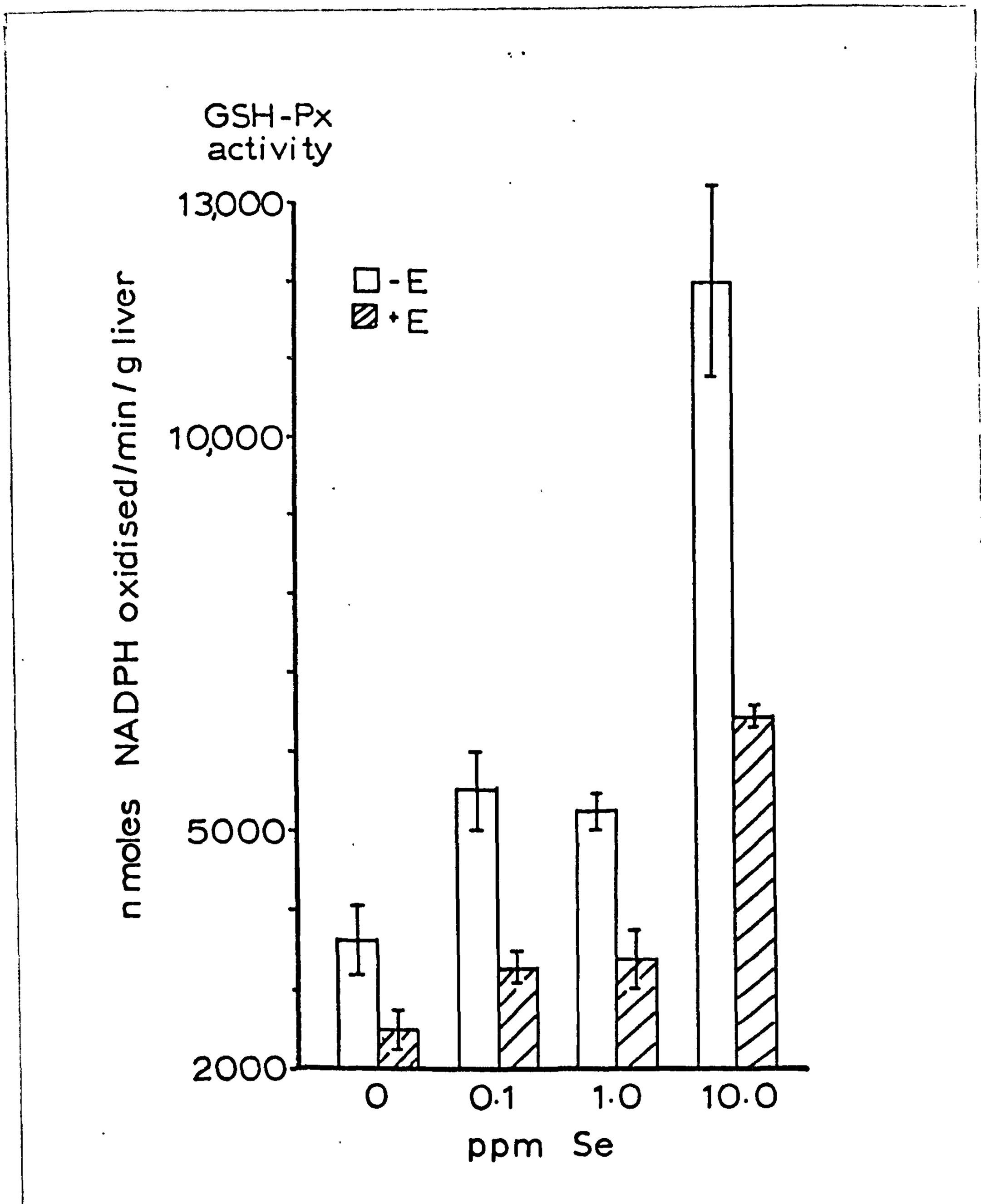
EFFECT OF DIETARY SILVER AND SELENIUM ON LIVER CYTOSOLIC GSH-Px ACTIVITY IN VITAMIN E-ADEQUATE RATS.

UNITS: n moles NADPH oxidised per minute per g liver.

Fig. 15-3.

The selenium- and vitamin E- deficient Torula yeast diet was given to weanling rats; half the animals received a supplement of 100mg α-tocopherol per kg diet and levels of selenium ranging from 0 to 10ppm were administered to all the animals. Rats were killed after 20-26 days and 100,000g supernatant fractions of liver were prepared and tested for glutathione peroxidase activity.

Bars represent the mean $\pm$ S.D of triplicate determinations on three livers.



EFFECT OF DIETARY VITAMIN E ON LIVER CYTOSOLIC GSH-Px ACTIVITY AT VARIOUS DIETARY LEVELS OF SELENIUM.

these animals were lower than those of animals which were not given vitamin E - supplemented diets. Thus addition of vitamin E to the diet led to a decrease in liver cytosolic GSH-Px activity.

III. Effect of dietary silver, selenium and vitamin E on the selenium content of rat liver:

Samples of liver tissue were washed in 0.25M sucrose solution and blotted dry before weighing. Triplicate determinations were carried out on each liver using about 0.5g liver for each analysis.

The selenium assay used was the fluorimetric measurement of the piazselenol complex with 2, 3 - Diaminonaphthalene; further details are given in Chapter 10.

Figure 15 - 4 shows the effect of dietary selenium and silver on liver selenium. The open bars represent those animals not given silver in their diet and increasing levels of dietary selenium in these rats led to increased liver selenium content, up to the highest level of dietary selenium studied. At 1.0 ppm, the concentration of selenium found in the liver was about 1.5μg/g liver. A ten-fold increase in the level of dietary selenium to 10 ppm increased liver selenium by a factor of 8 to 12.4μgSe per g liver.

The effect of silver in selenium-supplemented rats, was to decrease liver selenium. The closed bars in Figure 15 - 4 show lowered selenium content in livers of those animals which were fed 1000 ppm silver. The extent of the decrease rises with the increase in dietary selenium; thus at 0.1 ppm Se, the significance of the effect of silver as evaluated by the Student's t test, was given by $p < .02$. At 1.0 ppm and 10 ppm dietary selenium, the effect of silver was greater and the significance of the decrease was $p < .01$.

Fig 15-4 THE EFFECT OF DIETARY SELENIUM AND SILVER ON Se CONTENT OF LIVER IN VITAMIN E - ADEQUATE RATS.

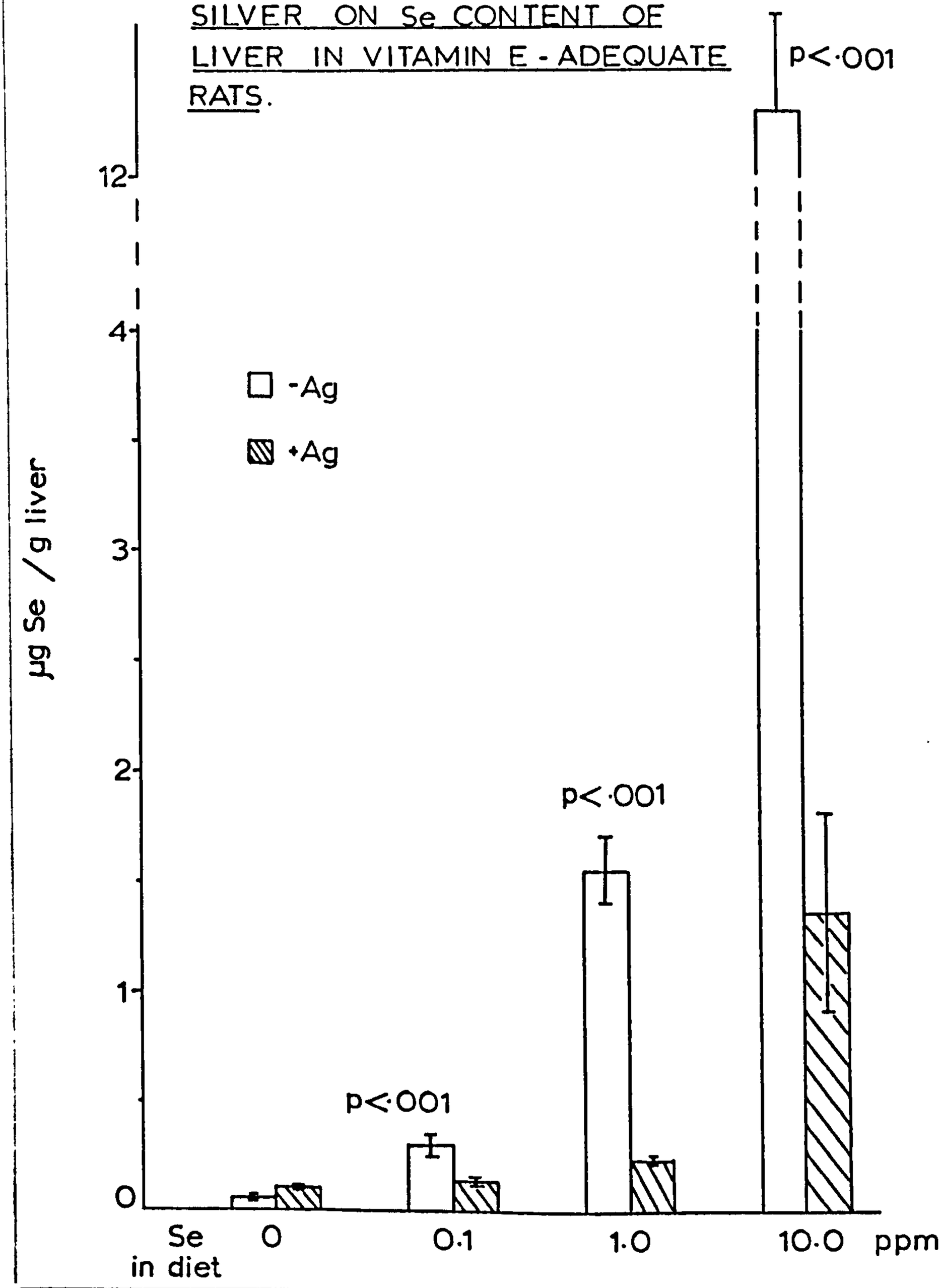


Fig. 15-4. Male weanling rats were maintained on the basal Torula yeast diet supplemented with 100mg ∝-tocopherol per kg, for 20-26 days. In addition, half the rats received 1000ppm silver as silver acetate in the diet and different levels of selenium ranging from 0 to 10ppm were administered in the drinking water. Selenium in liver tissue was measured by fluorimetric assay using 2,3-Diaminonaphthalene. The significance of the difference from deficient rats due to selenium administration was calculated by the Student's t test.

Fig. 15-5

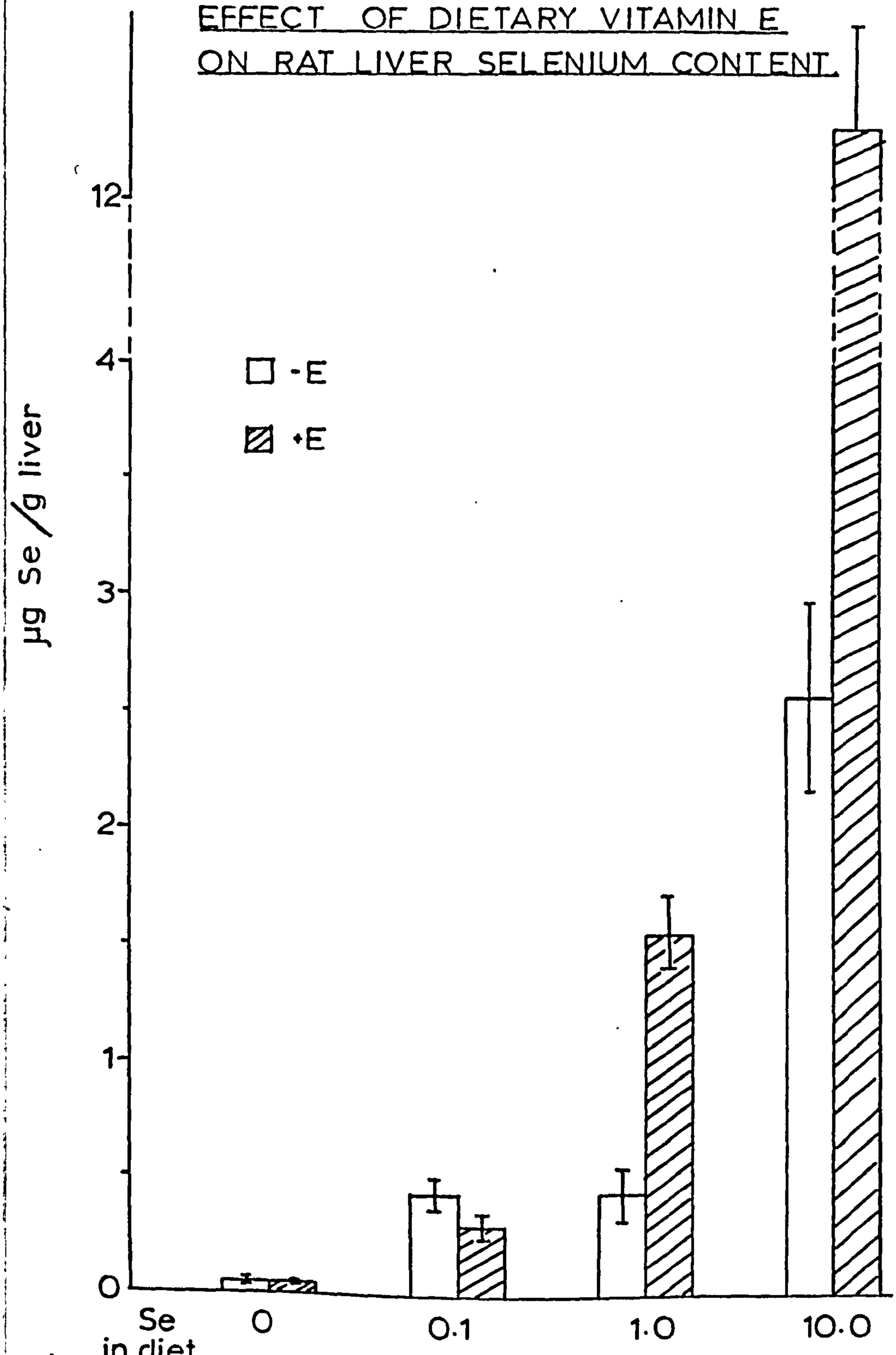


Fig.15-5. Male weanling Wistar rats (40-50g) were given the selenium- and vitamin E- deficient Torula yeast diet or that diet supplemented with 100mg ∝-tocopherol per kg diet. Levels of selenium ranging from 0 to 10ppm were a administered in the drinking water and animals were killed 20-26 days after commencement of the dietary treatments.

Liver selenium was measured by the fluorimetric method described in Chapter 10.

Effect of vitamin E:

The administration of 100 mg α- DL - tocopherol per kg of diet caused varied effects on liver selenium which were dependent on the level of selenium in the diet (Fig. 15 - 5). At low levels of dietary selenium (0 and 0.1 ppm Se), the addition of vitamin E to the diet either caused no change in liver selenium or resulted in a slight decrease which, when analysed by the Student's t test, was not statistically significant. When the dietary level of selenium was increased to 1.0 ppm and 10 ppm, supplementation of the diet with vitamin E caused an increase in the selenium content of liver which was statistically significant at both levels of dietary Se:- at 1.0 ppm, $p < .01$; at 10 ppm Se, $p < .001$ for the effect of vitamin E.

IV. Discussion:

The effects of silver and selenium on indices of selenium metabolism in vitamin E - deficient rats have been presented in Chapter 14. The experiments in this Chapter were designed to examine the possible effects of vitamin E on these particular parameters which might explain the nature of the interactions between vitamin E, selenium and silver.

The activity of the major detoxification route for acute and subacute doses of selenium was little affected by the presence of vitamin E when the level of dietary selenium presented to the animal was low. Earlier experiments using vitamin E - deficient animals (Chapter 14), showed that the administration of toxic levels of selenium induced or 'switched on' this pathway. Thus, at dietary levels of zero and 0.1 ppm Se, the activity of the dimethylselenide pathway was low, and the presence of vitamin E in the diet would not be expected to have a great effect on $DMSe$ generation. However, when the level of selenium supplementation was increased to 10 ppm, there was a six

hundred percent induction in the activity of the pathway in the presence of dietary vitamin E while the activity in vitamin E - deficient rats was increased by only about two hundred percent. Thus, in vivo vitamin E - supplemented rats would be expected to cope more effectively with toxic doses of selenium, since the excretory pathway for those animals would undergo greater stimulation than animals maintained on vitamin E - deficient diets. This protective effect of vitamin E against selenium toxicity was discussed in Chapter 13, when the survival period of rats, given dietary α - tocopherol and 20 ppm selenium, was shown to be much longer ($p < .02$) than animals given 20 ppm Se only.

The activity of the selenoenzyme, glutathione peroxidase, was shown in the last Chapter to increase with increasing dietary selenium; it therefore reflected the quantity of free selenium available to the animal for anabolic incorporation in various seleno-proteins. This finding confirmed that of Smith et al (1974) who demonstrated increased GSH-Px activity when rats were given higher levels of Se in their diets. Figure 15 - 3 shows that the presence of vitamin E in the diet led to a decrease in the activity of glutathione peroxidase which was statistically significant ($p < .02$) at 10 ppm dietary selenium. The sensitivity of this enzyme to dietary selenium observed in both this chapter and the previous one indicates that this decrease in enzyme activity may be due to a decreased availability of selenium in the presence of α - tocopherol, since vitamin E stimulates selenium excretion at this level.

Examination of the third parameter in selenium metabolism, the selenium content of liver, showed that administration of dietary vitamin E led to an increase in the concentration of selenium in the

liver at 10 ppm dietary Se. This result might at first seem to be at variance with the decreased activity of GSH-Px which was observed in these animals. However, it must be remembered that liver is a major excretory organ for selenium, and is responsible for the conversion of chronically toxic does of selenium to the trimethyl selenium ion $\left((CH_3)_3\ Se^+\right)$ which is excreted via the urine. Furthermore Moxon (1937), in a study on dogs, showed that accumulation of selenium occurred in liver and kidneys and that the concentration of the element in these organs was usually severalfold greater than in other organs.

The effect of dietary selenium (0 to 10 ppm) and silver (1000 ppm) on the selenium content of liver in vitamin E - supplemented rats was very similar to that observed in vitamin E - deficient animals. (Chapter 14). Increasing levels of selenium led to increased Se in liver and the administration of silver to Se-supplemented rats caused the level of selenium in the liver to decrease ($p < .02$). If, as it is suggested (Diplock et al, 1967), silver complexes free selenium in the tissues, this observed effect of silver in the presence or absence of dietary vitamin E is an indication that the fluorimetric method which is used in Se determination detects free selenium only and not that which is complexed with silver. The experiments reported in Chapter 10 confirm that about 0.1% of silver selenide (Ag_2Se) was detected by this method while the recovery figure for sodium selenite (Na_2SeO_3) was between 96 and 104 percent.

CHAPTER 16

THE EFFECT OF DIETARY VITAMIN E, SELENIUM AND OTHER TRACE ELEMENTS ON THE UPTAKE AND DISTRIBUTION OF ^{75}Se - SODIUM SELENITE IN RAT TISSUES.

In the experiments to be described in this Chapter, high levels of silver, mercury and cadmium were administered to young rats together with varying levels of dietary selenium and vitamin E.

A. SILVER

Animals and feeding regimen

Sixty-four male, weanling Wistar rats (40-50g) were divided into eight groups. The animals in four groups were given diet T, the Torula yeast-based diet, while rats in the other four groups received the same diet supplemented with 100 mg α- tocopherol per kg diet (T + E); all the animals were supplied with tap water ad libitum. After one week, (from Day 8), rats were given either distilled water or sodium selenite solutions containing different levels of selenium to drink until termination of the experiment; the levels of selenium supplements are given in Table 16-1. From day 15, half the rats in each group received a daily oral dose of 1000 ppm silver given as silver acetate solution. On each of days 20, 21 and 22, each experimental animal was given 10 μCi ^{75}Se administered orally in 0.2 ml $Na_2{}^{75}SeO_3$ solution in distilled water. All rats were killed on day 23 and the following organs were removed for ^{75}Se measurements: liver, lung, kidney, heart, testis, brain and a sample of skeletal muscle taken from the hind leg. After weighing, a homogenate of each organ in 3.0 ml of 0.25 M sucrose solution was prepared using an Ultra-Turrax tissue homogeniser; this procedure facilitated and standardised the ^{75}Se radioactivity determination as the homogenates were easier to handle than wet tissue and they were all of equal volume.

TABLE 16-1

DIETS AND DOSAGE OF ANIMALS

DAYS 1 - 7

64 RATS

Diet T	T + E
32 rats	32 rats

Tap water supplied to all rats

DAY 8 ONWARDS TO END OF EXPERIMENT

	Selenium levels in drinking water (ppm)
Diet T (32 rats) :	0 ; 0.1 ; 1.0 ; 10.0
Diet T + E (32 rats) :	0 ; 0.1 ; 1.0 ; 10.0

(Total = 8 groups)

DAY 15 ONWARDS TO END OF EXPERIMENT

Daily oral dose of 1000 ppm Ag given to half the rats in each group.

DAYS 20, 21, 22

All rats given a single oral dose of 10 μCi ^{75}Se per rat per day.

DAY 23

All rats killed.

PRESENTATION OF RESULTS

The ^{75}Se content of each tissue was calculated in counts per minute (c.p.m) and divided by the weight of the tissue used in the preparation of the homogenate. Thus, the units of radioactivity used were c.p.m. per g tissue. Since each dietary treatment was given to four rats, the results from all four animals were pooled and averaged; standard deviations were also calculated.

Figure 16-1 shows the effect of dietary selenium, silver and vitamin E on the uptake of ^{75}Se by rat tissues after the three subcutaneous injections, each of 10 μCi ^{75}Se.

DISCUSSION

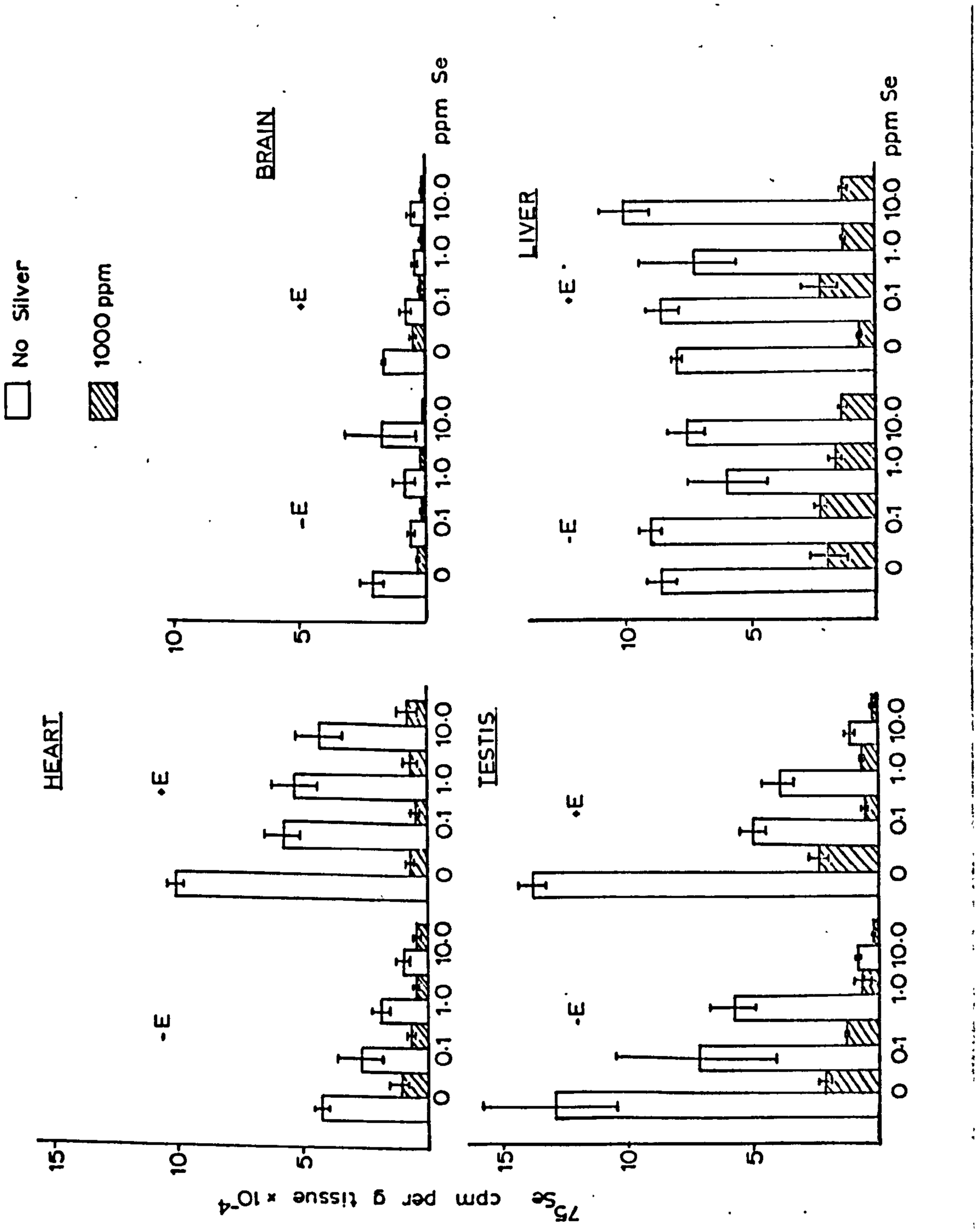
The amount of ^{75}Se retained by the tissues was in many tissues found to decrease as the concentration of selenium administered in the diet was increased. This was the pattern shown by heart, testis, lung and skeletal muscle and was thought to be due to the prevention of further uptake of the radioactive dose by adequate or high levels of dietary selenium. In brain, this pattern was apparent only in rats given the vitamin E-supplemented diet; the variability of the results from the vitamin E-deficient animals made it difficult to discern any pattern. Liver and kidney also did not show this inverse relationship between ^{75}Se retention and the dietary level of selenium; in particular, the amount of ^{75}Se found in kidney was high at all dietary levels of selenium. Both liver and kidney are excretory organs for selenium ,since excessive quantities of the element are disposed of as the trimethylselenonium ion through the urine (Dudley, 1936) or, at higher dose level, exhaled as dimethyl selenide (McConnell and Portman, 1952). Heinrich and Kelsey (1955) in studies on the distribution of selenium in the tissues of the mouse, gave weanling, female mice ^{75}Se (0.1 to 0.7 mg Se/kg) and found that the level of excretion via the liver reached a peak after one hour, (Fig. 2-1) whereas the urinary excretory pathway was employed at maximal

Fig. I6-I.

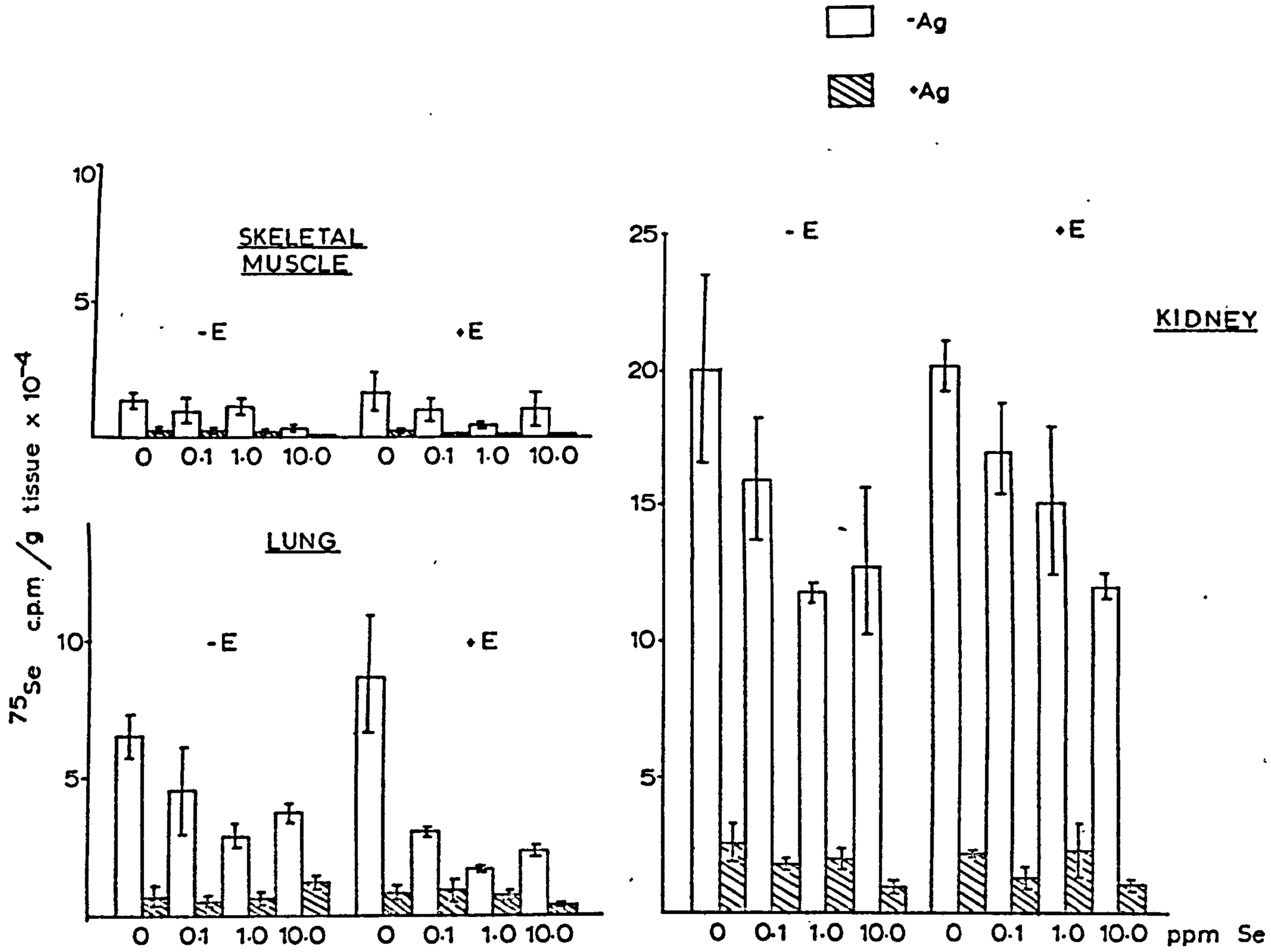
The effect of dietary silver, selenium and vitamin E on the uptake and distribution of ^{75}Se by rat tissues after the administration of 3 x IO Ci ^{75}Se.

The Torula yaest diet T or the same diet supplemented with I00 mg α-tocopherol per kg diet (T+E) was given to male, weanling rats for 23 days. Various levels of selenium ranging from 0 to I0 ppm were administered in drinking water from day 8 onwards. From day I5, a daily oral dose of I000 ppm silver was given to half the rats in each group. All rats received a subcutaneous injection of I0μCi ^{75}Se on each of days 20, 2I and 22. The experiment was terminated on day 23 when all the rats were killed; various organs were removed for ^{75}Se determination and the results were calculated as c.p.m. ^{75}Se per g tissue.

Each bar represents the mean $\pm$ S.D. of the results from four animals.



^{75}Se cpm per g tissue $\times 10^{-4}$


-Ag
•Ag
SKELETAL MUSCLE
-E
•E
10
5
0 0·1 1·0 10·0 0 0·1 1·0 10·0
LUNG
-E
•E
10
5
0 0·1 1·0 10·0 0 0·1 1·0 10·0
KIDNEY
-E
•E
25
20
15
10
5
0 0·1 1·0 10·0 0 0·1 1·0 10·0 ppm Se
^{75}Se c.p.m /g tissue x 10^{-4}

capacity forty-eight hours after administration of the ^{75}Se. Heinrich and Kelsey (1955) also measured the concentration of ^{75}Se in various tissues and found that, after forty-eight hours, liver and kidney retained the greatest amounts of the administered dose. The results presented here confirm those of Heinrich and Kelsey and show also that testis has a large requirement for selenium since, in the absence of dietary selenium, a high level of ^{75}Se was retained by the tissue.

EFFECT OF DIETARY SILVER

Silver consistently lowered the amount of ^{75}Se taken up by the tissues, irrespective of the level of dietary selenium and vitamin E and in all the tissues examined. The suggestion that silver complexes selenium in the tissues, probably to form silver selenide (Ag_2Se) has been discussed earlier in Chapters 14 and 15; the complexed selenide would thus be unavailable to the tissues for the biosynthesis of glutathione peroxidase and other seleno-proteins. Therefore, the administration of dietary silver to rats receiving high levels of dietary selenium would lead to the formation of relatively large amounts of silver selenide, which cannot be metabolised further. The presence of this in the tissues would be expected to be more inhibitory to further uptake of ^{75}Se than would high but metabolically active levels of dietary selenium.

EFFECT OF VITAMIN E

Vitamin E did not alter the distribution of ^{75}Se in brain, testis, liver, kidney, lung or skeletal muscle; in heart, however, vitamin E increased the amount of ^{75}Se retained by the tissue at all levels of dietary selenium in the absence of silver. It thus appears that vitamin E exerts a profound influence on the uptake of selenium by cardiac tissue.

Edwin *et al* (1961) determined vitamin E levels in various tissues of six month old vitamin E-deficient rats; when compared with brain or

liver, i.e. those tissues in which the administration of dietary vitamin E did not affect ^{75}Se uptake and retention (Fig. 16-1), the α- tocopherol content of heart was low (2.3 μg/g tissue), while that of brain was 3.1 μg/g and in liver it was 3.0 μg/g. Supplementation of the diet with DL - α - tocopheryl acetate (0.1 mg/g) increased the tocopherol levels in all three tissues (Edwin et al, 1961), but while that of brain tissue increased to 6.3 μg/g, representing a two-fold increase, and that in liver increased seven-fold to 21.3 μg/g, the tocopherol content of heart tissue increased nearly twenty-fold to 34.1 μg/g. These figures show that heart tissue has a particular requirement for vitamin E and the results presented in Fig. 16-1 extend this observation and show that vitamin E can also cause an increase in the uptake of selenium by the heart.

B. MERCURY

Animal diets and dosage

Sixty-four male, weanling Wistar rats (40-50g) were divided at random into eight groups; the animals in four groups were given the Torula yeast-based diet T, while rats in the other groups received the vitamin E-supplemented diet, T + E, as previously described. All the animals were given tap water to drink for one week, after which either distilled water or sodium selenite solutions containing different levels of selenium were supplied. Twenty days after the start of the experiment, the animals were weighed and a single subcutaneous injection of mercury (II) chloride (containing 0.01 mM Hg/Kg body weight) was administered to half the rats in each group. In addition, on each of days 20, 21 and 22, all animals were given 10 μCi ^{75}Se in 0.2 ml sodium ^{75}Se-selenite solution. The experiment was terminated on day 23 and rats were killed by gassing them with carbon dioxide; various organs were then removed for ^{75}Se determination.

RESULTS AND DISCUSSION

The effects of dietary selenium, mercury and vitamin E on the uptake and retention of ^{75}Se by testis, heart, lung, liver and kidney are shown in Fig. 16-2. If the open bars alone are considered (i.e. those animals to which mercury was not administered), the general effect of increasing levels of dietary selenium was to decrease the amount of ^{75}Se which was taken up by the tissues. This finding was not surprising as the same effect was observed in Fig. 16-1 and it indicates that adequate or high levels of dietary selenium prevented the uptake of further selenium by the tissues.

EFFECT OF MERCURY:

At all levels of dietary selenium, and in the presence or absence of dietary vitamin E, the administration of mercury decreased the retention of ^{75}Se by the tissues. The greatest effect was observed in testis and kidney while heart and liver tissue from vitamin E-supplemented rats showed moderate responses. However, in vitamin E-deficient animals, the administration of mercury had little or no effect on the uptake of ^{75}Se by heart and liver and lung tissue was affected only when the level of dietary selenium was low (0 ppm).

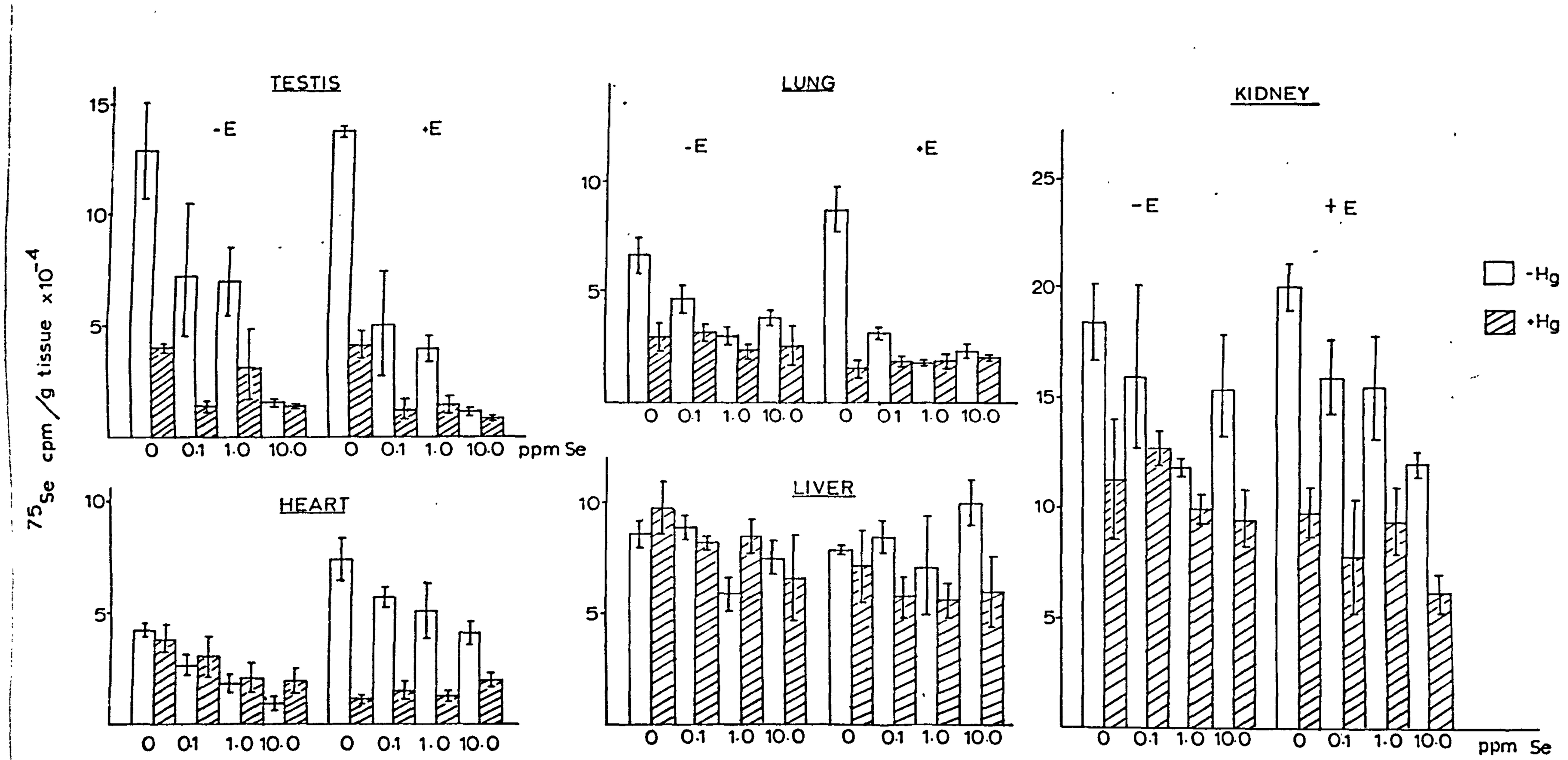
Dietary protection by selenium against mercuric chloride toxicity in rats has been observed by Parizek et al (1969; 1969a) and by Potter and Matrone (1974); in addition, Ganther et al (1972) observed a decrease in toxicity of methyl mercury when added to diets containing selenium. Yamane et al (1977) reported a significant increase in the mercury content of liver from rats given $HgCl_2$ with selenium as compared with rats receiving $HgCl_2$ only. The liver selenium content was also greater in rats given both Se and Hg than for rats given Se only; the molar ratio of mercury to selenium was 1:1 and it was suggested (Yamane et al, 1977) that selenium interacted directly with mercury to form less toxic products in the liver. In addition, Komiya et al (1977) have

Fig. I6-2.

The effect of dietary selenium, mercury and vitamin E on the short-term uptake of ^{75}Se by rat liver, lung, testis, heart and kidney.

Animals were given either diet T or diet T+E throughout the experiment as described in the text. Various levels of selenium, ranging from 0 to I0 ppm, were supplied in drinking water from day 8 until the termination of the experiment. A single subcutaneous injection of 0.0I mM Hg per kg body weight was administered on day 20 to half the animals in each group. On days 20, 2I and 22, each rat received an oral dose of I0μCi ^{75}Se per day in 0.2 ml sodium ^{75}Se-selenite solution. the experiment was terminated on day 23 and various organs were removed for ^{75}Se determination.

Each bar represents the mean $\pm$ S.D. of the results from four animals.


TESTIS
LUNG
KIDNEY
HEART
LIVER
-E
+E
-Hg
+Hg
0 0.1 1.0 10.0
ppm Se
^{75}Se cpm/g tissue x10^{-4}

verified the presence of a complex in rat blood plasma and erythrocytes, formed by equimolar proportions of mercury and selenium and HgSe-protein complexes have been identified by gel-chromatographic separation of plasma proteins.

Thus, in Fig. 16-2, a decrease in ^{75}Se uptake shown by tissues from rats given mercury, is consistent with the increased selenium levels found by Yamane et al (1977) in rats given $HgCl_2$ and selenium; since the administration of mercury resulted in increased retention of selenium from the diet, this would lead to prevention of further uptake of ^{75}Se by the tissues.

EFFECT OF VITAMIN E

The administration of dietary vitamin E (100 mg DL - α - tocopherol per kg diet) led to increased ^{75}Se uptake in heart tissue. These results confirmed those obtained in Fig. 16-1 and were thought to be due to the specific requirement for vitamin E demonstrated by Edwin et al (1971) for heart tissue.

C. CADMIUM

Animals and feeding regimen

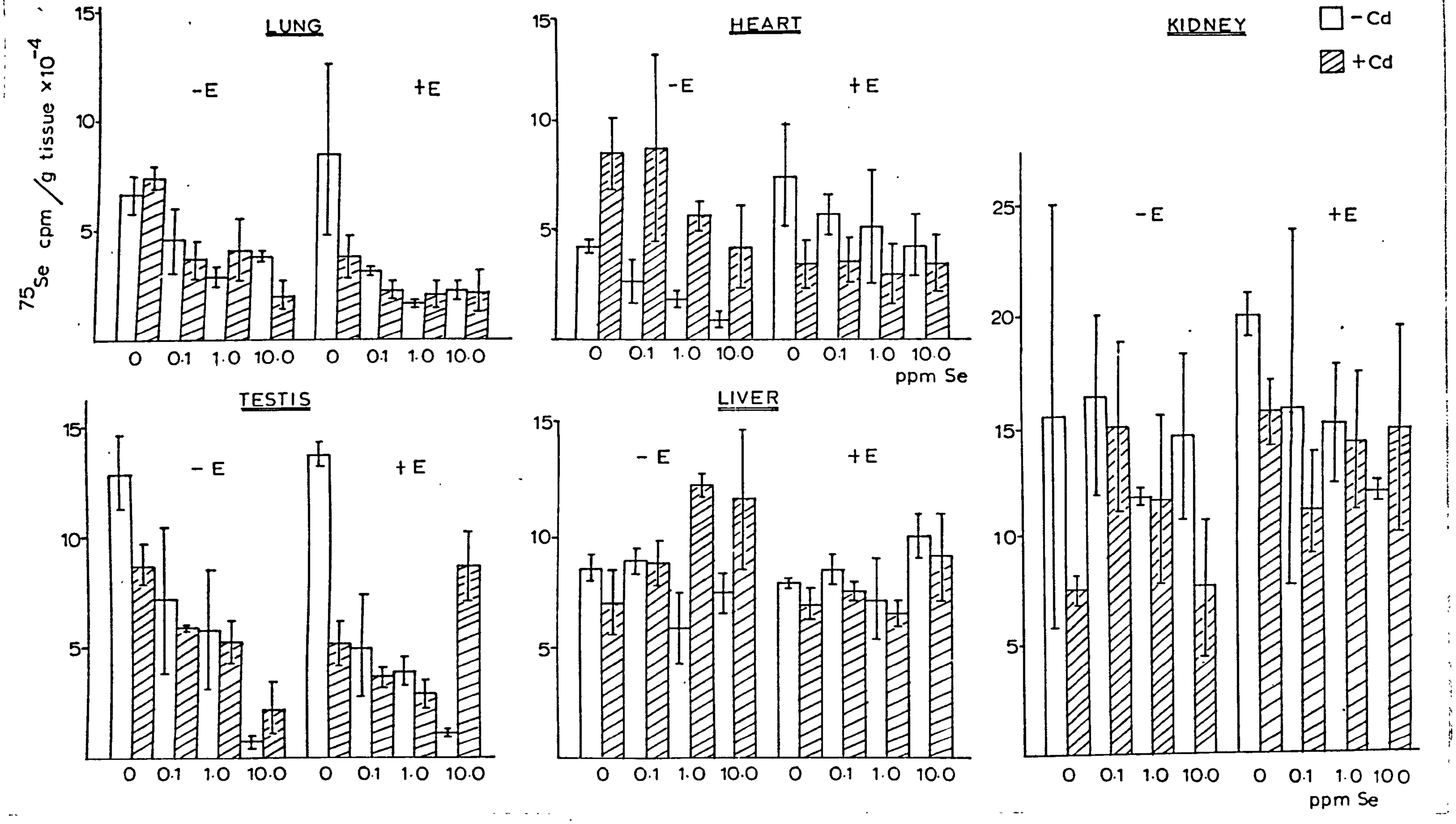
Sixty-four male, weanling Wistar rats (40-50g) were divided into eight groups; the animals in four groups were given the Torula yeast-based selenium - and vitamin E-deficient diet (T) while those in the other groups were fed the same diet supplemented with 100 mg α - tocopherol per kg diet (T + E). On the eighth day after the start of the experiment, the rats were given either distilled water or sodium selenite solutions containing 0.1 to 10 ppm Se as drinking water. Cadmium was administered to half the animals in each group on day 20 by a single subcutaneous injection containing 0.01 mM Cd per kg body weight. An oral dose of 10 μCi ^{75}Se was given to all the animals on days 20, 21 and 22 and the experiment was terminated on day 23. Rats were killed by carbon dioxide gassing and various organs were removed for ^{75}Se determination.

Fig. I6-3.

Uptake and distribution of ^{75}Se in various tissues of rats given dietary selenium, vitamin E and cadmium.

Weanling rats were given the selenium deficient diet (T) or that diet supplemented with vitamin E (T+E). After one week, various levels of selenium ranging from 0 to IOppm, were supplied in drinking water. Twenty days after the start of the experiment, 0.0ImM Cd/kg body weight, was administered as a single subcutaneous injection to half of the rats in each group; ^{75}Se was given in three oral doses, each of IOμCi ^{75}Se, on days 20, 2I and 22. Rats were killed on day 23 and the levels of ^{75}Se retained by testis, heart, lung, liver and kidney were determined.

Each bar represents the mean $\pm$ S.D. of the results from four animals.


LUNG
HEART
KIDNEY
TESTIS
LIVER
-E
+E
- Cd
+Cd
^{75}Se cpm /g tissue x10^{-4}
0 0·1 1·0 10·0 0 0·1 1·0 10·0
ppm Se

RESULTS AND DISCUSSION

The results of the effect of dietary selenium and cadmium on the uptake and distribution of ^{75}Se are shown in Fig. 16-3. The general effect of increasing levels of dietary selenium was to decrease the amount of ^{75}Se retained by the tissues, except in the excretory organs kidney and liver.

EFFECT OF CADMIUM

There was no clear indication of the effect of cadmium on the short-term uptake of selenium by rat tissues. Subcutaneous administration of cadmium had greatest effect in heart where, in the absence of dietary vitamin E, cadmium increased ^{75}Se retention at all levels of dietary selenium studied; when vitamin E was included in the diet (100 mg α- tocopherol per kg diet), cadmium decreased the level of ^{75}Se retention by heart tissue.

The effect of dietary vitamin E was, as in the experiments described previously, most marked in heart tissue. The administration of tocopherol increased the retention of ^{75}Se at all levels of dietary selenium. Further, dietary vitamin E decreased the level of selenium which was retained when cadmium (0.01 mM $CdCl_2$/kg body weight) was administered by subcutaneous injection.

Cadmium has been shown (Parizek and Zahor, 1956) to cause testicular necrosis in rats and this toxic effect can be prevented by the administration of zinc (Parizek, 1957) and of selenium (Kar et al 1960); Mason et al, 1964; Mason and Young, 1967). In addition to its effect on cadmium toxicity in testis, selenium is capable of preventing all other known manifestations of cadmium toxicity. Thus, selenium decreased the mortality of rats given lethal doses of cadmium (Parizek et al, 1968; Gunn et al, 1968). It is of interest to note in Fig. 16-3, that at toxic levels of dietary selenium (10 ppm Se), the subcutaneous administration of cadmium resulted in an increase in ^{75}Se retention by

some tissues, suggesting that the level of selenium which was available to these tissues was considerably lowered in the presence of cadmium and hence the uptake of further selenium. The ability of cadmium to interact with selenium would thus explain the effect observed by Parizek et al (1968) of decreased cadmium-induced mortality, when selenium was administered to rats.

The increased uptake of ^{75}Se in heart tissue from vitamin E-deficient rats following the administration of cadmium, was reversed by the addition of vitamin E to the diet; this observation suggested the involvement of a specific function of α- tocopherol in protecting the tissue against peroxidation. Thus, if cadmium acts to remove 'free' selenium from tissues, the level of selenium biologically available for incorporation into seleno-proteins, would be lowered when cadmium was administered. The activity of the seleno enzyme glutathione peroxidase would thus decrease in the presence of dietary cadmium and the observed increase (Fig. 16-3) in ^{75}Se uptake after cadmium-treatment could be a response to the need to synthesize more GSH-Px in order to prevent oxidative damage by peroxides. In the presence of dietary vitamin E, however, this 'need' would not be so great because tocopherol would protect the tissues against peroxidation.

PART IV

GENERAL DISCUSSION

The major part of this thesis has been concerned with investigating the effect of various dietary treatments on the metabolism of selenium compounds. The knowledge that between 18% and 20% of the total selenium present in normal mammalian tissues exists as selenide (Diplock et al, 1973), and that inorganic selenium compounds can be converted to organic metabolites, led to further investigations into the pathway for the biosynthesis of organo-selenium compounds. The pathway chosen for study was the synthesis of dimethyl selenide from sodium selenite, a major metabolic route for detoxifying subacute doses of selenium.

A. SYNTHESIS OF DIMETHYL SELENIDE BY LIVER FRACTIONS

Experiments undertaken to determine the ability of various subcellular fractions to catalyse the synthesis of dimethyl-selenide revealed that the unit of activity used in the calculations was a very important factor in measurements of the relative activity of the fractions. Thus, when expressed in terms of the weight of liver contained in each fraction, the post-microsomal (S_2) and post-mitochondrial (S_1) supernatant fractions had similar activities which were higher, than the activities of the washed microsomal fraction (M_s) and of a 1 : 1 (v/v) combination of S_2 and M_s. In similar experiments, Ganther (1966) expressed the activities of subcellular fractions in terms of the

protein content of the fractions, and he reported that the combination of post-microsomal supernatant fraction (S_2) and washed microsomal fraction (M_s), had the highest activity of those tested, while the post-microsomal supernatant by itself was least active.

The difference between the results reported in this thesis (Chapter 8) and those obtained by Ganther was attributed to the different units used in the calculations and two major criticisms of Ganther's work were made:

(1) In an early paper on the synthesis of dimethyl selenide, Ganther (1966) defined the unit of specific activity as "the number of mμmoles of selenium volatilized per minute per mg. of protein." In the same publication, it was shown that the rate of DMSe formation was not constant and that an initial lag phase preceded a period of rapid activity. Therefore, consideration of the rate of DMSe synthesis must indicate at which stage, during the incubation, the rate was determined.

(2) The use of the protein content of liver subcellular fractions in calculations of their activity (Ganther, 1966) was thought to be injudicious, owing to the nature of the method by which the fractions were prepared. The size and shape of a protein or other subcellular

particle, on which separation by centrifugation is based, bears no relationship to its activity in the synthesis of dimethyl selenide. Therefore, another unit of activity was defined which would relate to the weight of tissue used in the preparation of subcellular fractions, and would not be affected by variations in centrifuging techniques.

B. INFLUENCE OF VITAMIN E AND SELENIUM ON DMSe GENERATION

Preliminary experiments were carried out to determine the optimum conditions under which liver fractions would catalyse the synthesis of dimethyl selenide. Following this, the effect of dietary vitamin E and/or selenium on the synthesis of dimethyl-selenide was investigated. The results showed that if selenium is included in a diet which is deficient in vitamin E, selenium will enhance its own metabolism in the liver. When vitamin E was administered, the stimulatory effect of selenium was either not observed or was greatly diminished. Thus, the vitamin appears to have a modulating effect on selenium metabolism.

C. SELENIUM AND GLUTATHIONE PEROXIDASE

Following the discovery (Rotruck et al, 1973) that selenium is an integral part of the enxyme glutathione peroxidase, measurements of the activity

of this enzyme were included in studies of selenium metabolism. The assay procedure used was a modification of the method described by Paglia and Valentine (1967); the post-microsomal supernatant fraction of rat liver was used as the source of glutathione peroxidase and the effect of various dietary treatments on the activity of the enzyme within this fraction was taken to be typical of the enzyme in the rest of the liver; (it is also found within the mitochondria). The enzyme assay involved measurement of the rate of utilisation of NADPH which was used to reduce glutathione (GSSG) which had been oxidised during the reduction of cumene hydroperoxide. In preliminary experiments, the optimum concentration of the peroxide substrate was determined and the molar extinction coefficient of NADPH was measured. All subsequent calculations of the activity of glutathione peroxidase were based on the extinction coefficient thus obtained.

D. DETERMINATION OF SELENIUM IN TISSUES

An attempt was made to develop an analytical method for the determination of small quantities of selenium in rat tissues. The method chosen involved the use of 2,3-Diaminonaphthalene (one of a group of aromatic o-diamine compounds used for this purpose) as a complexing reagent for Se (IV). The piazselenol thus formed is brightly coloured and can be measured fluorimetrically; in all experiments carried out using

this method, as little as 0.01 μg Se could be detected and the fluorescence of the complex was linear with Se concentration over the range 0.1 to 6 μg Se per ml solution. Recovery experiments carried out showed that between 95% and 101% of the selenium content of a sample was recovered even after digestion with nitric acid and perchloric acids. The fluorimetric method was shown to be highly specific for Se (IV) compounds and an average of 102% of selenium present as sodium selenite was detected while the comparable figure for sodium selenate was less than 1%. Experiments in Chapters 13-16 describe the use of silver salts as a dietary stress factor in vitamin E-deficient rats; Diplock et al (1967) suggested that silver lowers the biological availability of selenium, possibly by forming a complex such as Ag_2Se with selenium. It was thus useful to know the limits of detection of silver selenide using the fluorimetric method described above; experiments using ^{75}Se-labelled hydrogen selenide showed that ca. 0.1% of selenium as silver selenide was detected by fluorimetric analysis.

E. SILVER TOXICITY, SELENIUM AND VITAMIN E

The effect of silver on selenium- and vitamin E-deficient rats and on indices of selenium metabolism was investigated. Silver was shown to be toxic to vitamin E-deficient rats and increasing levels of selenium were found to be partially protective against silver toxicity; dietary vitamin E was also found to

protect against silver toxicity. When the generation of dimethyl selenide was examined, silver was shown to decrease DMSe generation at high levels of dietary selenium whether or not vitamin E was included in the diet. In addition, at high levels of dietary selenium (10 ppm), silver lowered the activity of the seleno-enzyme glutathione peroxidase in the liver; analysis of the selenium content of liver showed that dietary silver caused a decrease in liver selenium, possibly by the formation of silver selenide.

Experiments on the uptake of selenium by rat tissues showed that silver consistently lowered the amount of ^{75}Se-sodium selenite retained by several tissues after the administration of three consecutive daily doses of 10μc_i ^{75}Se.

F. <u>THE EFFECT OF CADMIUM AND MERCURY ON THE DISTRIBUTION OF SELENIUM IN TISSUES</u>

The uptake and retention of ^{75}Se by testis, heart, lung, kidney and liver of rats given dietary selenium, vitamin E and either mercury or cadmium was investigated. The administration of mercury decreased ^{75}Se retention by the tissues, at all levels of dietary vitamin E and selenium. The greatest effect was observed in testis and kidney while heart and liver tissue from vitamin E-supplemented rats showed moderate responses.

The subcutaneous administration of cadmium had

greatest effect on heart tissue where, in the absence of dietary vitamin E, cadmium increased ^{75}Se retention at all levels of dietary selenium studied. In the other tissues, no clear indication of the effect of cadmium on ^{75}Se uptake and retention was obtained.

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The Influence of Dietary Vitamin E and Selenium on the Metabolism of Selenium by Rat Liver

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Previous work has established the presence in rat liver of acid-volitile, protein-bound Se, whose existence, depends on vitamin E in the diet (1). Subsequently (6) the acid-volatile Se was shown to be selenide and it was suggested (2, 6) that reduction of SeO_3^{2-} to Se^{2-} might require α-tocopherol in the tissues, to participate in the reduction or to protect oxidant-sensitive Se. The reduction of toxic amounts of Se proceeds (7) in liver by a stepwise sequence involving GSH and GSH reductase, followed by methylation of a reactive selenide by a methyl transferase and S-adenosyl methionine. The first part of the pathway (7) may also be responsible (2) for metabolism of trace amounts of Se destined for utilisation of Se in its metabolic roles e.g. in glutathione peroxidase (8) or in non-haemironproteins (3). In liver the methyl transferase is present (7) in the microsomal fraction and in kidney it is localised in the soluble fraction. The work described here was a study of the possible effects of dietary vitamin E and Se on methylation of ^{75}Se in rat liver and an examination of whether these dietary factors affected intracellular localisation of Se metabolising enzymes.

Experimental

Animals and Diets

Weanling male Wistar rats (50-60 g) were given the vitamin E-deficient diet (5) or that diet supplemented with 100 mg/kg DL-α-tocopheryl acetate, or the vitamin E- and selenium-deficient diet (Diet T) (5) or that diet with either 0.1 ppm Se (Diet T + Se), or with 100 mg/kg DL-α-tocopheryl acetate (Diet T + E), or with both Se and α-tocopheryl acetate (Diet T + E + Se).

Homogenisation and Centrifugation

20 % liver homogenates were prepared using the technique (1) designed to minimise variability in homogenisation. Homogenates were centrifuged at 9,000 g for 10 min. and the supernatant fraction was re-centrifuged at 100,000 g for 1 hr. The 100,000 g pellet was re-suspended in 0.25 M sucrose, and re-centrifuged at 100,000 g for 1 hr; the pellet, called microsomal fraction, was resuspended in 0.25 M sucrose. Dimethyl selenide (DMSe) generation. Formation of $(CH_3)_2\ ^{75}Se$ from $Na_2\ ^{75}SeO_3$ was studied as described (6); $(CH_3)_2\ ^{75}Se$ generation was calculated from the loss of ^{75}Se from the reaction vial in 30 min. A unit of activity was defined arbitrarily as the percentage

loss of ^{75}Se from the reaction vial expressed per g of liver. The actual loss of ^{75}Se from the vial was around 20 %.

Estimation of Glutathione Peroxidase (GSHpx)

The method of Paglia and Valentine (9) with cumene hydroperoxide as substrate was used; units used were moles NADPH oxidised/min/g liver.

Results and Discussion

Table 1. Experiment 1. Vitamin E and dimethyl selenide (DMSe) generation in rat liver

Fraction	Vitamin E-deficient		Vitamin E-supplemented	
	Expt. A	Expt. B	Expt. A	Expt. B
9,000 g	126 ± 79	114 ± 50	79 ± 0	99 ± 5
100,000 g	95 ± 5	98 ± 13	87 ± 3	69 ± 3
100,000 g + Microsomes	137 ± 40	117 ± 10	108 ± 2	112 ± 9

The diets were given for 7 months. Values given are Mean Units DMSe ± S.D. of 3 observations on the liver of each of 3 rats.

Experiment 1, DMSe generation in the liver 9,000 g fraction of vitamin E-deficient rats appeared higher (Table 1) than when vitamin E was present, but this could not be established statistically because of the variability of the results from the vitamin E-deficient rats. The activity of the 100,000 g fraction was lower ($P>0.01$) than of the 9,000 g fraction, and activity was enhanced by the addition of the microsomal fraction.

Experiment 2 investigated the effect of prior treatment of rats with Se on metabolism of ^{75}Se. In the absence of vitamin E, selenium metabolism was profoundly increased by the inclusion in the diet of 0.1 ppm Se (Fig. 1). However, when 100 mg/kg of vitamin E was added, selenium metabolism was not significantly different from that in the control rats. Thus, vitamin E appears to have a modulating influence on the metabolism of Se.

The mechanism by which vitamin E exerts its influence on selenium metabolism is obscure. Previous experiments (5) showed that the presence of protein-bound selenide in rat liver depended upon the presence of vitamin E in the diet. We have suggested (4) that the biological role of vitamin E may be to stabilise membranes in vivo, partly by inhibiting peroxidation among polyunsaturated fatty acyl moieties of membrane phospholipids, and partly by a physical interaction of the α-tocopheryl side-chains with arachidonyl residues of membrane phospholipids. If our suggestion (6) is valid

that the same pathway is responsible for the metabolism of selenium to the immediate precursor both of dimethyl selenide and of physiological selenium - containing proteins, then it is possible that vitamin E acts to divert selenium from the excretory pathway to the physiological pathway when supply of dietary selenium is limited. It can be further speculated that this control could be mediated via the microsomal membrane since the methylation of the intermediate selenol is achieved (7) in liver by a microsomal trans methylase. Alternatively, tocopherol might act by modulating the synthesis of the GSHpx apoprotein, or by controlling the insertion of selenium into the apoprotein. In preliminary experiments we have found that the liver soluble GSHpx level in rats given Diet T + Se was 343 ± 17 units/g, which was significantly lower ($P>0.001$) than in rats given Diet T + E + Se (471 ± 44 units/g), although addition of α-tocopherol <u>in vitro</u> raised the apparent level somewhat in the rats given Diet T + Se.

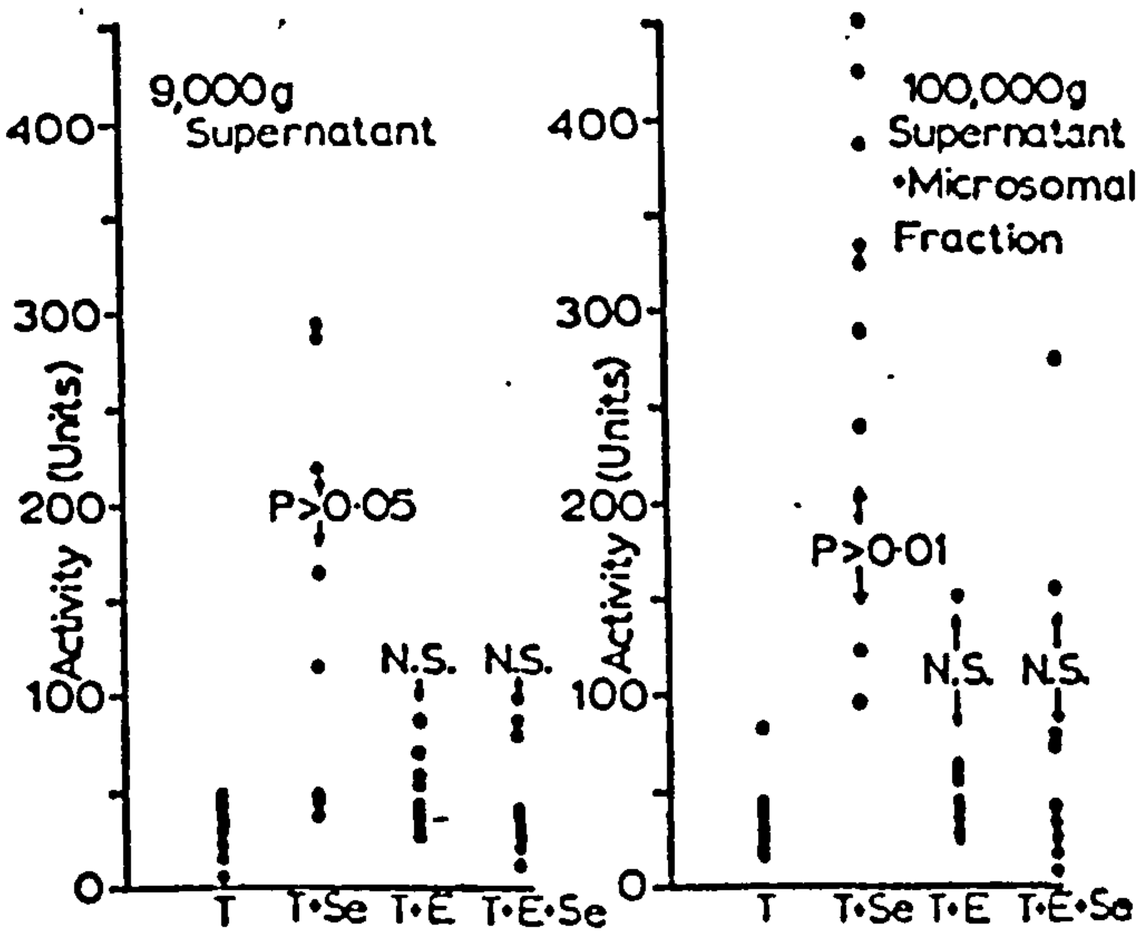

FIGURE 1. EXPERIMENT 2. EFFECT OF DIETARY VITAMIN E AND SELENIUM OF THE GENERATION OF DIMETHYL SELENIDE BY RAT LIVER SUBCELLULAR FRACTIONS

RATS WERE GIVEN THE VITAMIN E - AND SELENIUM - DEFICIENT TORULA YEAST DIET (T), OR THAT DIET SUPPLEMENTED WITH 0.1PPM SE (T + SE), OR 100MG/KG VITAMIN E (T + E), OR BOTH SE AND E (T + E + SE) FOR 20-26 DAYS. TECHNIQUES USED FOR PREPARATION OF FRACTIONS AND DMSE GENERATION ARE GIVEN IN THE TEXT. EACH POINT REPRESENTS A SINGLE DETERMINATION MADE ON THE LIVER OF ONE RAT. STATISTICAL EVALUATION OF THE DIFFERENCES BETWEEN THE CONTROL AND THE SUPPLEMENTED GROUPS WAS BY THE STUDENT'S TEST.

Summary

Formation of dimethyl selenide (DMSe) by a rat liver cell-free system was significantly enhanced by prior treatment of the rats with selenium, provided that vitamin E was absent from the diet. Addition of vitamin E to the diet modulated DMSe formation, perhaps by decreasing the activity of the microsomal transmethylase.

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